

THE RELATIONSHIP AMONGST STRESS, TEMPERAMENT, AND IMMUNE
FUNCTION IN BRAHMAN CATTLE

A Dissertation

by

NICOLE CASSANDRA BURDICK

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2010

Major Subject: Physiology of Reproduction

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ABSTRACT

The Relationship Amongst Stress, Temperament, and Immune Function in Brahman
Cattle.

(August 2010)

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The studies described herein were designed to determine the influence of temperament on stress hormones and the immune system in response to various stressors. These stressors included transportation, lipopolysaccharide (LPS) challenge, and adrenocorticotropin (ACTH) challenge. In the first transportation study, bulls (8 Calm, 8 Intermediate, and 8 Temperamental) were loaded into a trailer and transported for 9 hr. Rectal temperature (monitored via indwelling recorders) increased within 0.5 hr of transportation, with greater peak rectal temperature in Temperamental than Calm bulls. Pre- and post-transport concentrations of cortisol and epinephrine were not affected by transportation, but were greater in Temperamental than Calm bulls. A second transportation study utilized 2 automatic sampling devices to allow the recording of rectal temperature and collection of blood samples, respectively. Rectal temperature was not affected by transportation or temperament in response to 4-hr of transport. Average heart rate oscillated between 60 and 130 bpm in Temperamental bulls, but remained around 100 bpm in Calm bulls. Transportation did not affect concentrations of

epinephrine, although concentrations were greater in Temperamental bulls than Calm bulls. Cortisol concentrations increased in Calm bulls but not in Temperamental bulls in response to transportation. Additionally, there were limited effects of transportation on peripheral blood mononuclear cell proliferation, IgM production, and cytokine gene expression. Specifically, proliferation tended to be greater post-transport. Expression of the glucocorticoid receptor was, and the expression of toll-like receptor 4 tended to be, reduced post-transport, as analyzed by quantitative real-time RT-PCR. In a study utilizing a LPS challenge, basal stress hormone concentrations during the pre-challenge period were greater in Temperamental bulls than Calm bulls. However, in response to the LPS challenge, only the epinephrine response was influenced by temperament. Additionally, Temperamental bulls exhibited a smaller increase in rectal temperature and sickness behavior than Calm bulls. In the last study, change in gene expression in peripheral blood mononuclear cells in response to acute increases in cortisol was assessed. Plasma cortisol and gene expression of cytokines and the glucocorticoid receptor tended to increase in response to placement of jugular cannula. Additionally, administration of ACTH significantly increased plasma concentrations of cortisol and the gene expression of some cytokines (interleukin-4 and interleukin-10). This suggests that acute increases in cortisol may have positive effects on immune function in Brahman calves. Through an increased understanding of the interaction between the stress response and animal temperament, as well as how stress hormones and temperament influence immune function, animal management practices can be modified to reduce negative impacts on growth and productivity.

DEDICATION

To my parents, Bill and Brenda, and my boyfriend, Roel, for your unwavering love and support. You constantly encouraged me, never doubted my abilities, and have helped me reach the height of my potential. I know that I would not be where I am today without you in my life. I love you all very deeply.

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CHAPTER I

INTRODUCTION

Stressors encountered by animals can pose economical problems for the livestock industry due to increased costs to the producer as well as the consumer. An increase in the stress hormones in response to management procedures such as weaning, castration, and transportation can inhibit growth, reproduction, and immune functions. The behavioral response of livestock to handling can also greatly affect management and production as wilder cattle can increase the risk of injury to both the cattle and the producer. Temperament is defined as the reactivity of cattle to humans and novel environments. More temperamental or wilder cattle have greater basal concentrations of stress hormones, decreased growth rates and average daily gain, and weaker immune responses to pathogens. Stress hormones have been demonstrated to inhibit many functions of the immune system, thus leaving animals more susceptible to disease and infection. Through an increased understanding of the interactions between the stress response and animal temperament, as well as how stress and temperament influence immune function, animal management practices can be modified to reduce negative impacts on growth and productivity.

CHAPTER II

LITERATURE REVIEW

STRESS

Hans Selye was the first author in the biomedical field to use the term stress, a term previously used in the field of physics. In his landmark 1936 article in *Nature*, Selye described a physiological triad that is common to all stressors: 1) thymicolymphatic involution, 2) adrenal enlargement, and 3) gastric ulceration (Selye, 1936). In 1939 Walter Cannon further clarified the definition of stress by defining the term homeostasis; yet, Cannon never actually used the term “stress” (Cannon, 1939; Pacak and Palvoits, 2001). Selye alluded to the phenomenon of homeostasis in his article in which he termed the efforts of an organism to return to homeostasis as the “general adaptation syndrome” (Selye, 1936). However, Selye focused on responses of the adrenal cortex, mainly the production of cortisol, while Cannon was more interested in the sympathetic nervous system’s role in the stress response (Cannon, 1939; Pacak and Palcovits, 2001). It is now well understood that both systems have roles in the body’s response to stressors, which will be discussed in more detail in later sections. Several researchers have challenged Selye’s theory of non-specific responses to stressors, suggesting that different types of stress (i.e., foot shock versus cold stress) produce different responses (Pacak, 2000; Pacak and Palcovits, 2001). For example, Pacak and Palcovits (2001) suggest that immobilization stress induces greater increases in adrenocorticotropin (ACTH) than catecholamines, while cold stress induces greater

increases in norepinephrine than ACTH or glucocorticoids. While the responses to certain stressors may be characterized by specific physiological responses, the physiological triad described by Selye 80 years ago remains a well-known response to chronic stress today.

The classical definition of stress used by Selye has been modified in order to reflect increases in our current knowledge base regarding the subject. For our current research, stress is defined as “a state in which homeostasis is actually threatened or perceived to be so; homeostasis is re-established by a complex repertoire of behavioral and physiological adaptive responses of the organism” (Chrousos and Kino, 2005). Stress is stimulated by a stressor and affects the body through activation of the hypothalamic-pituitary-adrenal axis (HPA axis) and the sympathetic nervous system (more specifically the sympathomedullary system; Butcher and Lord, 2004). Stressors are any internal or external stimuli or threat (physical, psychological, or chemical) that disrupts homeostasis (Aguilera, 1998; Black, 2002). In response to this altered state, the stress response is activated in order to help the body deal with the threat and return to or maintain homeostasis.

Hypothalamic-Pituitary-Adrenal (HPA) Axis

In response to stressful stimuli, higher brain centers stimulate neurons in the paraventricular nucleus (PVN) of the hypothalamus, resulting in the synthesis and secretion of corticotrophin-releasing hormone (CRH) and vasopressin (VP; Carrasco and Van de Kar, 2003). The major source of CRH and VP that induces changes in

corticotrophs in the anterior pituitary is from the release of CRH and VP from neurons into the median eminence (Aguilera, 1998). Binding sites for CRH are also found outside the hypothalamus including in the brain stem, adrenal medulla, heart, liver, kidneys, and testes (Tsigos and Chrousos, 2002). Upon stimulation by CRH, corticotrophs within the anterior pituitary synthesize and secrete ACTH into the circulation (Carrasco and Van de Kar, 2003; Webster Marketon and Glaser, 2008). Via endocrine mechanisms ACTH stimulates the production of glucocorticoids by the adrenal cortex (Makara et al., 1981; Carrasco and Van de Kar, 2003). In mammals the primary glucocorticoid is cortisol. However, rodents lack the enzyme P450c17, which is responsible for producing cortisol in the adrenal, and therefore their primary glucocorticoid is corticosterone (Ashwell et al., 2000). Upon adrenocortical stimulation by ACTH, glucocorticoids are released and distributed via the circulatory system and act systemically to produce a variety of effects depending on 1) the amount of hormone secreted, 2) the duration of secretion, 3) the tissue which they elicit their effect, and 4) the amount of glucocorticoid receptors present in the target tissue. Glucocorticoids are also responsible for negative feedback on both the hypothalamus and the pituitary by inhibiting synthesis and/or secretion of CRH, expression of the CRH receptor, VP secretion into the median eminence, and the secretion of ACTH (Aguilera, 1998).

In the absence of stressor stimulation, CRH and ACTH, and therefore cortisol, are released in a circadian, pulsatile manner, approximately 2 to 3 times per hr, which is greatest in the early morning (Tsigos and Chrousos, 2002). The release of CRH and ACTH can be modulated or altered due to changes in lighting, feeding schedules,

activity, and ultimately stress (Tsigos and Chrousos, 2002). In nocturnal animals, such as rodents, the circadian rhythm of secretion is reversed (i.e., cortisol is greater after dusk in early evening; Ashwell et al., 2000).

Glucocorticoids are transported through the circulatory system by carrier proteins that prevent degradation and allow glucocorticoids to be soluble. Carrier proteins also allow glucocorticoids to be available quickly after induction of the stress response. Albumin is the major carrier protein for cortisol, but it can also be transported by cortisol binding globulin (transcortin). Approximately 1-10% of cortisol circulates as a “free” steroid (Ashwell et al., 2000; Rhen and Cidlowski, 2005). Tissues also have the ability to increase or decrease available cortisol within cells through 11 β hydroxysteroid dehydrogenase (HSD11 β) enzymes, which convert cortisone to cortisol (type I) and vice versa (Type II; Rhen and Cidlowski, 2005).

Glucocorticoids can bind to both the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). The MR has a greater affinity for glucocorticoids than the GR. When concentrations of glucocorticoids are high enough to fully saturate the MR, glucocorticoids will bind to the GR, resulting in many of the actions of glucocorticoids on the HPA axis and other body systems (Carrasco and Van de Kar, 2003). The MR is found in some limbic brain areas such as the hippocampus as well as the heart, kidney, and colon. In contrast, the GR is necessary for life and is ubiquitously expressed within the body (Carrasco and Van de Kar, 2003; Smoak and Cidlowski, 2007). There are two major GR variants: GR- α and GR- β (Löwenberg et al., 2008; Smoak and Cidlowski, 2007). The GR- β does not have the ability to bind glucocorticoids as it is missing the C-

terminus of the protein (i.e., ligand binding domain; Ashwell et al., 2000). However, it has the ability to bind to the GR antagonist, RU486, and is believed to act as a negative regulator of GR- α (Smoak and Cidlowski, 2007). The GR- α variant is the classical glucocorticoid receptor, and will be referred to as GR for the remainder of this review.

The GR is a cytoplasmic receptor that remains inactivated by heat shock proteins 90, 70, 23, and immunophils (Ashwell et al., 2000; Charmandari et al., 2005).

Following the binding of glucocorticoids to the cytoplasmic GR, the receptor undergoes a transformational change resulting in the exposure of the nuclear translocation signal (Sapolsky et al., 2000). Once in the nucleus the GR will bind to glucocorticoid response elements (GRE) and negative GREs (nGRE) on DNA, allowing for the direct regulation of gene expression. Additionally, the GR can inhibit transcription through protein-protein interactions with transcription factors, regulate signaling through membrane associated receptors and second messengers, and also has the ability to change the stability of mRNA molecules in cells and the electrical potential of neuronal cells (Tsigos and Chrousos, 2002; Rhen and Cidlowski, 2005).

The binding of glucocorticoid to the GR results in the modulation of gene expression (increase or decrease transcription) of numerous genes, with the effects being tissue-specific. Genes related to immune function that are specifically regulated by glucocorticoids, which will be described in more detail in later sections, include both pro- and anti-inflammatory cytokines, prostaglandin synthesis enzymes, and cell adhesion molecules (Besedovsky and del Rey, 1996; Sapolsky et al., 2000).

Prostaglandin synthesis enzymes are important for blood vessel remodeling to allow for

vasodilation and diapedesis of leukocytes (squeezing of leukocytes between two endothelial cells). Cell adhesion molecules allow for the initial binding of leukocytes to the endothelial cells and for the strong binding that precedes diapedesis.

Sympathomedullary System (SMS)

The sympathetic nervous system is activated in response to many stressors in parallel to and often prior to the stimulation of the HPA axis. Upon stimulation, noradrenergic neurons in the brain and postganglionic sympathetic neurons innervating peripheral organs (e.g., heart, vasculature, kidneys, gut, and adipose) secrete norepinephrine into the circulation, resulting in increased blood pressure, heart rate, and respiration rate. Additionally, nerve impulses in high cortical centers within the brain relay messages through the limbic system resulting in the release of norepinephrine, serotonin, and acetylcholine, which activate the PVN (Black, 2002). In conjunction with these actions, preganglionic sympathetic fibers innervating the adrenal medulla stimulate the production and secretion of epinephrine and norepinephrine via acetylcholine (Butcher and Lord, 2004). The proportion of epinephrine to norepinephrine secreted can vary by animal species. In most mammals, including humans and cattle, a majority of the catecholamine secreted from the adrenal medulla is epinephrine, with limited norepinephrine. Therefore, norepinephrine concentrations measured in circulation are due to secretions by postganglionic sympathetic neurons. An additional subset of vagal and sacral parasympathetic efferent nerves are activated which mediate the gut response to stress (Tsigos and Chrousos, 2002).

There are two major types of catecholamine receptors, the α - and β -adrenergic receptors. Both have several subtypes and are all membrane-bound receptors. The β - and the α_2 -adrenergic receptors are G-coupled protein receptors, while the α_1 -adrenergic receptor stimulates increases in intracellular calcium through phosphatidylinositol (Madden et al., 1995). These receptors are differentially expressed in tissues and elicit a wide range of effects. For example, the β_2 -adrenergic receptor is well-distributed in the body and is the prominent receptor subtype found on lymphocytes of the immune system. Additionally, the effects of norepinephrine are mainly mediated by the α_2 -adrenergic receptor (Van der Poll, 2000). A change in the density of adrenergic receptors has been implicated in several autoimmune diseases (Madden, 2003).

The sympathetic nervous system regulates many functions in the body including the cardiovascular, gastrointestinal, respiratory, and renal systems, all of which can be modulated in response to SMS activation (Charmandari et al., 2005). An increase in epinephrine concentrations in the brain serves as an alarm system, resulting in a decrease in neurovegetative activities (e.g., eating and sleeping) and the activation of the stress response (HPA axis activation; Tsigos and Chrousos, 2002). The secretion of norepinephrine within the brain also activates the fear behaviors and enhances long term memory and storage of adversely charged emotions in the hippocampus (Sapolsky et al., 2000; Tsigos and Chrousos, 2002).

Interactions Between the HPA Axis and the SMS

The responses of the HPA axis and the SMS to stress are highly concordant. In response to most stressors both systems are activated and have the ability to synergistically activate each other. For example, reciprocal connections exist between the norepinephrine and CRH systems in the brain, allowing for each hormone to activate the other (Tsigos and Chrousos, 2002). The release of norepinephrine, serotonin, and acetylcholine in the brain stimulates the PVN, resulting in the secretion of CRH (Black, 2002). In this manner norepinephrine can modulate the release of ACTH and subsequently glucocorticoids (Chrousos and Gold, 1992). Similarly, CRH can stimulate the locus coeruleus, a dense collection of autonomic neurons in the brainstem, to secrete norepinephrine (Black, 2002). Also, GRs are present in sympathetic neurons which allow glucocorticoids to regulate the synthesis, uptake, and tissue content of norepinephrine (Chrousos and Gold, 1992). Glucocorticoids can modulate the expression of the β -adrenergic receptor both through genomic (via the GR) and non-genomic means (Janssens et al., 2008). Together, catecholamines and glucocorticoids increase cardiovascular output and catabolic effects (e.g., metabolism of glycogen, protein, and triglycerides to provide energy), yet inhibit many body systems, including reproduction and immunity (Chrousos and Gold, 1992; Butcher and Lord, 2004).

The ability of an animal to perceive a situation as stressful depends on prior experiences and developmental history. Both combine to either sensitize or protect the animal from particular challenges (McEwen et al., 1997). Behavioral responses to challenges are different depending on the individual, resulting in aggression, submission,

humiliation, or adaptation. These responses will either increase or decrease the vulnerability of the animal to subsequent challenges (McEwen et al., 1997).

Furthermore, the stress response of an individual may protect it against certain immune challenges, yet make it susceptible to others (McEwen et al., 1997). In cattle, the behavioral response to humans, as well as novel environments, has been linked to stress responsiveness. These differences in behavioral responses have been demonstrated to alter immune functions in cattle.

TEMPERAMENT

Stress has been linked to certain human behaviors and conditions, including fear, anxiety, and depression (Tyrka et al., 2008). In cattle, changes in behavior, such as their fear response to humans and/or to novel environments, is defined as temperament (Fordyce et al., 1988a). In recent years the secretion of stress hormones has been linked to temperament in many species including cattle and mice (Sorensen et al., 2005; Curley et al., 2008). For example, mice that over-express phenylethanolamine-n-methyl transferase (PMNT), the enzyme that converts norepinephrine to epinephrine, produce greater amounts of epinephrine and are more aggressive (Sorensen et al., 2005). A study in humans suggested that cortisol secreted in breast milk may influence infant temperament (Glynn et al., 2007). In cattle, differences in temperament have been linked to stress responsiveness with more excitable (Temperamental) cattle having greater basal concentrations of cortisol than Calm cattle (Fell et al., 1999; Curley et al., 2006; King et al., 2006).

Temperament can be measured by several methods, with the two used most often being exit velocity (flight speed) and pen score. Exit velocity (Burrow et al., 1988; Curley et al., 2006) is an objective measurement defined as the rate (m/s) at which an animal traverses a specified distance after exiting a working chute. Pen score (Hammond et al., 1996) is a subjective measurement of temperament in which cattle are separated into groups of three to five animals, and are ranked on a scale of 1 to 5 based on their reactivity to a human observer. An animal scored as a 1 is described as being very calm and approachable while an animal scored as a 5 is described as being very volatile and aggressive. Together exit velocity and pen score can be averaged to assign a temperament score. Based on temperament score, cattle can be ranked into temperament groups (e.g., Calm, Intermediate, and Temperamental). Exit velocity has been correlated with serum concentrations of cortisol in cattle. Specifically, cattle with greater measurements of exit velocity have greater basal concentrations of cortisol (Fell et al., 1999; Curley et al., 2006).

Temperament has also been linked to decreased performance (weight gain and average daily gain) of cattle. For example, cattle with slower exit velocities gain weight faster than those with faster exit velocities (Burrow and Dillon, 1997; Müller and von Keyserlingk, 2006). Additionally, more nervous cattle have been reported to have a greater carcass bruising or bruise score when compared to calmer cattle (Fordyce et al., 1985; Fordyce et al., 1988b). Therefore, cattle with excitable temperaments have been suggested to increase production costs due to the increase risk of injury and a decrease in

carcass value (Burrow, 1997). Similar to stress, temperament has also been suggested to negatively affect immune function, as detailed in a later section.

IMMUNE FUNCTION

A healthy immune system is necessary to maintain livestock production. A greater incidence of sickness has been demonstrated to decrease growth, leading to increased days on feed and ultimately to greater costs of production and decreased profits. Morbidity and mortality due to disease, particularly bovine respiratory disease, is the most significant health problem facing the beef cattle industry (Duff and Galyean, 2007). Additionally, Wittum et al. (1994) found a 15.9 kg reduction in weaning weight due to calf morbidity. Diseased cattle increase live weight cost due to the costs of death loss, treatment, and decreased feed intake which ultimately results in lower live weight gains (Larson, 2005). Steers that displayed lung lesions at slaughter have been found to have lighter hot carcass weight, lower dressing percentage, less internal fat, and lower marbling scores (Larson, 2005). Clearly, diseased cattle cost producers a substantial amount of money each year. Therefore it is essential to find methods to 1) quickly identify cattle that are showing signs of sickness, 2) quickly and appropriately treat sick cattle in order to minimize ill-effects of sickness on feed intake and growth, and 3) develop methods to minimize the occurrence of disease in cattle. In order to accomplish these tasks it is essential to gain a better understanding of the immune system and how the body responds to pathogens.

The immune system is separated into two distinct branches: the innate and the adaptive immune systems. The innate immune system is the first line of defense against invading pathogens and involves cells that have general, non-specific receptors that are able to identify self and non-self molecules. In contrast, the adaptive or acquired immune system has greater specificity and is able to elicit specific responses against invading pathogens. Both will be discussed in more detail below.

Innate Immunity

The innate immune system is the more ancient of the two branches as it is the only immune system in less developed organisms. This system involves both physical components and barriers, including the skin, mucosal tissues, and the gut, and cellular components including macrophages, neutrophils, dendritic cells, natural killer cells, and mast cells which have receptors that detect infection or tissue damage (Barton, 2008). Complement, a group of plasma proteins that bind to and kill pathogens, is also part of the innate immune system (Männel, 2007). The detection of an antigen by the innate immune system results in the initiation of the inflammatory response to help remove the pathogen from the body. The cells of the innate immune system do not have receptors that recognize specific antigens, as in the adaptive immune system, and therefore elicit a non-specific response. However, they are able to discriminate self versus non-self by recognizing certain classes of molecules on pathogens that are not typical of that particular species (Männel, 2007).

Pathogens are recognized by cells of the immune system, specifically macrophages, neutrophils, and dendritic cells, which express pattern recognition receptors or PRRs. These receptors recognize portions of pathogens named pathogen associated molecular patterns or PAMPs. Examples of PAMPs include lipopolysaccharide (LPS) of gram negative bacteria, peptidoglycan of gram positive bacteria, and double stranded RNA which is often found in viruses (Janeway, 2005; Storni et al., 2005). Examples of PRRs are toll-like receptors (TLR; plasma membrane receptors), nod-like receptors (NLRs; cytosolic receptors), and RIG-I-like receptors (cytosolic receptors; Barton, 2008). These receptors initiate signaling cascades ultimately resulting in pro-inflammatory cytokine secretion, and activation of the adaptive immune system (Barton, 2008). In one example, activation of TLR-4 results in increased secretion of the cytokines interferon- β (IFN- β), tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), IL-6, and IL-13 (Eicher et al., 2004). Cytokines released in response to antigen recognition also enhance the inflammatory response through vasodilatation of blood vessels and eventually help activate the adaptive immune system.

The best studied of the PRRs are the TLRs. Toll receptors were originally found in *Drosophila*, where they regulate embryonic dorsal-ventral pattern (Leon et al., 2008). The carboxyl region of the receptor is very similar to the vertebrate IL-1 receptor (Leon et al., 2008). As they were discovered, similar receptors in other species were named toll-like receptors (Werling and Jungi, 2003). The TLRs are part of the IL-1 receptor/TLR superfamily. They have leucine-rich repeats in the extracellular domain and an intracytoplasmic region containing a region with TOLL/IL-1 receptor homology

(Werling and Jungi, 2003; Leon et al., 2008). The TLRs can bind several different molecules including LPS (TLR-4), peptidoglycan (TLR-2), flagellin (TLR-5), double-stranded RNA (TLR-3), and CpG DNA (TLR-9; Werling and Jungi, 2003). Upon binding of PAMPs to TLRs, a signaling cascade ensues resulting in the production and release of reactive oxygen and nitrogen intermediates (e.g., nitric oxide and superoxide anions), activation of the adaptive immune system, increases in pro-inflammatory cytokines and co-stimulatory molecules, and the maturation and migration of dendritic cells to draining lymph nodes (Werling and Jungi, 2003). One of the most recognized and studied PAMPs is LPS. The interaction of LPS with TLR-4 involves a complex of other molecules including CD-14 (cluster of differentiation-14), LPS binding protein (LBP), and MD-2 (Werling and Jungi, 2003). In cattle, TLR-2 and -4 are present on dendritic cells, macrophages, and monocytes, which is similar to humans (Werling and Jungi, 2003). However, in contrast to humans, there is no difference in the number of mRNA transcripts for these two TLRs in cattle (Werling and Jungi, 2003).

Upon detection of infection, neutrophils are the first immune cell to migrate from circulation to the infected area, followed by monocytes (Barton, 2008). Neutrophils produce granules which contain cytotoxic enzymes and molecules that cause microbial lysis, but can also damage healthy cells and tissue in the area if released in excessive amounts. Neutrophils and monocytes (macrophages upon activation and differentiation within tissues) secrete many pro-inflammatory cytokines that initiate the inflammatory cascade. Pro-inflammatory cytokines, $\text{TNF-}\alpha$, $\text{IL-1}\beta$, and lipid mediators dilate blood vessels in the area and cause the blood vessels to become “leaky,” allowing for an

increase in plasma flow and lymphocyte migration to the infected area (Barton, 2008). Neutrophils are present in the infected tissue for a limited time, and undergo apoptosis followed by removal by macrophage phagocytosis (Barton, 2008). Macrophages are also involved in the phagocytosis and degradation of pathogens, mainly bacteria. Once the infection is contained, a shift to an anti-inflammatory response is initiated to reduce inflammation and support tissue repair (Barton, 2008). This shift is vital to prevent a hyper-inflammatory state, which can result in more tissue damage and injury than the initial infection itself, potentially leading to sepsis, septic shock, and death (Zhao et al., 2008). However, the signals that initiate the shift from pro-inflammatory to anti-inflammatory are still under investigation.

In response to inflammation, tissue damage, and infection, the body initiates an acute phase response (APR). The APR is a set of reactions that promote tissue damage repair, control of invading organisms, wound healing, and/or recruitment of host defense mechanisms (Black, 2002). This includes the production of acute phase proteins from the liver, and the production of cytokines, catecholamines and, to a lesser extent, glucocorticoids. The APR is also characterized by physiological responses within the animal including fever and sickness behavior (Black, 2002). This response usually subsides within 24 to 48 hr following stimulation (Black, 2002).

Cytokines

Cytokines are the main activating molecules of the innate and adaptive immune system, and have been called the hormones of the immune system. They are small

proteins, approximately 80 kDa or smaller, and have a high potency at low (picomolar) concentrations (van der Poll, 2000). These molecules function to direct the proliferation, growth, and differentiation, as well as specific actions of cells within the immune system (Elenkov, 2008). Cytokines are often separated into two categories: pro-inflammatory and anti-inflammatory. Pro-inflammatory cytokines include TNF- α , IL-1 β and IL-6, and their actions can be regulated by soluble cytokine receptors (van der Poll, 2000). The production of TNF- α is the main mediator of sepsis, a condition that develops when the body has a decreased ability to respond to a pathogen, which usually results in excessive activation of the immune system (Pfeffer, 2003). In the absence of infection, injection of TNF- α can produce a physiological response similar to septic shock (Pfeffer, 2003). This cytokine is biologically active as a trimer, and its receptors belong to the TNF receptor family. The main receptor, TNF- α R1, has a 'death component' that results in the apoptosis of cells *in vitro* (Pfeffer, 2003). Additionally, TNF- α R1 is essential for surviving infections with intracellular bacteria (Pfeffer, 2003).

Anti-inflammatory cytokines serve to limit inflammation in order to prevent a hyper-inflammatory state, which, in the case of *E. coli* infection, can lead to sepsis, septic shock, and possibly death (Männel, 2007). An example of a prominent anti-inflammatory cytokine is IL-10. The role of cytokines in immune function, specifically inflammation, will be discussed in a later section.

The production of cytokines is not limited to immune cells. Cells within the pituitary can also secrete IL-6 in response to LPS stimulation as well as other mediators (Besedovsky and del Rey, 1996). Additionally, IL-1 β and IL-8 have been found to be

secreted by the pituitary, and other cytokines have been noted to be secreted by the adrenal gland, testes, ovary, the central nervous system, as well as the pancreas (Besedovsky and del Rey, 1996).

Adaptive Immunity

The adaptive immune system is activated by both cytokines and cells of the innate immune system, and is able to provide a stronger, more specific immune response to clear the pathogen from the body (Chaplin, 2006; Barton, 2008). Similar to innate immune cells, adaptive immune cells use cell surface receptors to recognize antigens in the body. In contrast to the innate immune system, adaptive immune cell receptors have high specificity for only one antigen.

The major cell types involved in adaptive immune responses are T lymphocytes (T cells) and B lymphocytes (B cells), each with several subtypes (Webster Marketon and Glaser, 2008). The receptor on T cells is called the T cell receptor (TCR), while the B cell receptor is surface-bound immunoglobulin. Both receptors are very similar in composition. Helper T cells (typically abbreviated as T_H), a subtype of T cells, are major players in the activation and development of the adaptive immune response. Once dendritic cells interact with an antigen in an infected tissue, they migrate from the site of infection, travel through the lymph system, and activate T_H cells in secondary lymphoid organs (i.e., lymph nodes and the spleen). Dendritic cells present antigens to T_H cells using major histocompatibility complexes (MHC). Class I MHC is present on all cells within the body except for red blood cells. Class II MHC is only present on

macrophages, dendritic cells, and B cells. The TCR on T cells recognizes antigen only when bound to a MHC molecule. Furthermore, in order for a T cell to be activated, it must receive co-stimulation. The recognition of antigen by a T_H cell, followed by co-stimulation, will result in the secretion of cytokines (Webster Marketon and Glaser, 2008). The T_H cells can be categorized based on the type of cytokine they produce (i.e., pro- versus anti-inflammatory). For example, T cells characterized by the production of $IFN-\gamma$ are labeled as T_H1 cells, and those that produce IL-4 and IL-5 are labeled as T_H2 cells (Männel, 2007). Activated T_H1 cells secrete IL-2 and $IFN-\gamma$, which will stimulate the differentiation of a second T cell subtype, cytotoxic T cells. The secretion of IL-2 and more specifically IL-4 and IL-5 by T_H2 cells will stimulate the activation and differentiation of B cells into plasma cells and memory B cells. Upon activation, these lymphocyte subtypes will clonally expand (i.e., expand only those lymphocytes with the specific receptor for the antigen) and will leave the secondary lymphoid organs via the circulatory system and travel to the site of infection. Cytotoxic T cells in the infected tissue release cytotoxic material causing degradation of the antigen. Similar to the granules of neutrophils, the contents of cytotoxic T cell granules can also cause lysis of healthy cells and tissues within the infected area. The activation of cytotoxic T cells and their subsequent actions to eliminate pathogens is called cell-mediated immunity (CMI).

Plasma cells secrete proteins known as immunoglobulins (Ig; antibodies) specific for the antigen, resulting in opsonization, complement fixation, or phagocytosis of the pathogen or products produced by the pathogen. These antibodies include classes IgA, IgD, IgE, IgG and IgM, with all but IgD being secreted. Additionally, each antibody

class has several subtypes. In cattle the most predominant immunoglobulin is IgG, specifically the subtype IgG₁. In mice, it has been established that cytokines modulate the antibody response to a pathogen (Estes et al., 2002). Similarly, there is evidence in cattle that certain cytokines can polarize antibody responses to certain subtypes (i.e, IFN- γ induces IgG₂ while IL-4 induces IgG₁ and IgE; Estes et al., 2002). The production of immunoglobulins and their subsequent influence on the immune response is called the humoral immune response (humoral immunity).

Cytokines produced by T_H cells continue to stimulate activation, differentiation, and migration of immune cells (both innate and adaptive) to the infected area. Eventually, the cytokine profile secreted by T_H cells will shift to an anti-inflammatory profile in order to promote and enhance termination of the immune response, clearance of debris, and tissue repair.

STRESS AND IMMUNE FUNCTION

The inflammatory response is one of the most primitive protective mechanisms, as fundamentals existed even before the development of the nervous system (Black, 2002). It is believed that the stress response evolved from, and is intricately linked to, the inflammatory response, as it is highly conserved over time and species, and the production of corticosteroids and catecholamines have the ability to stimulate the production of cytokines and acute phase reactants, similar to an inflammatory response (Black, 2002). The immune system evolved from the inflammatory system, and is therefore linked with the stress response. Additionally, the development of the

sympathetic nervous system *in utero* is in parallel with the development of neurogenic inflammation (Black, 2002).

It was first discovered in the 1940s that glucocorticoids had anti-inflammatory effects, much of which was a surprise to glucocorticoid physiologists at the time (Hench et al., 1949; Munck et al., 1984). Since this discovery, glucocorticoids have been used to treat many patients with inflammation and also to inhibit the immune system to allow successful organ transplantation (Sapolsky et al., 2000). The discovery of the anti-inflammatory actions of glucocorticoids also suggested that Selye was wrong in his logic that inflammatory diseases, such as rheumatoid arthritis, were caused by maladaptations of the stress system, as glucocorticoids were able to calm the diseases (Munck et al., 1984). However, studies since have demonstrated that products of the stress response (i.e., glucocorticoids) alone can cause inflammation, with excessive or chronic stress implicated in the development of inflammatory diseases (Black, 2002). Additionally, animals and humans with autoimmune diseases have been characterized as having greater basal concentrations of cortisol (Ashwell et al., 2000).

The immune system can be regulated by several different endocrine secretions, with the most prominent being those secreted in response to stress. Stress hormones can have both negative and detrimental effects, leaving the animal more susceptible to disease, as well as positive and preparative actions, which may help the animal more appropriately defend its body against pathogens (Avitsur et al., 2006). Whether the resulting effect is either positive or negative following exposure to a stressor is

dependent upon the duration and type of stressor, the timing of infection relative to the stressor, as well as which immune cells are targeted (Avitsur et al., 2006).

Stress alters immune function in a temporal manner subsequent to the binding of glucocorticoids to intracellular receptors within lymphocytes and other immune cells (Bauer et al., 2001). However, as stated above, this is dependent on the duration of stress. Acute stress, characterized as stress exerted for a short period of time, can stimulate immune function. In contrast, chronic stress, or stress exerted repeatedly or for an extended period of time, negatively impacts the ability of the immune system to fight off disease (Shi et al., 2003). In the following sections the negative effects of stress hormones on immune function will be discussed, followed by emerging research that describes positive effects of stress hormones on immune function.

Negative Effects of Stress on Immune Function

There is an inverse relationship of the diurnal changes in glucocorticoid concentrations and the number of circulating peripheral leukocytes (i.e., when cortisol is high, the number of circulating leukocytes is low, and vice versa) and individuals that lack an adrenal gland do not experience this diurnal change (McEwen et al., 1997). Studies indicate that mice with larger adrenal glands have smaller thymus glands (Cavigelli et al., 2007). This suggests that the greater output of cortisol by the adrenal gland negatively affects T lymphocyte maturation in the thymus, therefore negatively affecting adaptive immune function. Thymocytes (immature T cells) are much more sensitive to the effects of glucocorticoids compared to mature T cells (McEwen et al.,

1997). Additionally, the production of cortisol binding globulin is down-regulated during the acute phase response to a pathogen, thus increasing glucocorticoid availability (Black, 2002). In addition to decreasing the circulating number of leukocytes, glucocorticoids also inhibit cell adhesion molecules in both endothelial and immune cells, thus inhibiting the ability of leukocytes to leave the circulation and enter inflamed or infected tissue (Sapolsky et al., 2000).

There are many ways to quantify the effect of stress hormones on immune function including determining the extent of *in vitro* cellular proliferation as well as quantifying concentrations of immunoglobulins and cytokines both *in vivo* and *in vitro*. Mitogen-induced lymphocyte proliferation is often utilized as a model to study CMI (Bauer et al., 2001; Webster Marketon and Glaser, 2008). Studies have indicated that both acute and chronic stress reduce lymphocyte proliferation. Specifically, glucocorticoids have been demonstrated to induce apoptosis of immune cells (Ashwell et al., 2000; De Bosscher and Haegeman, 2009). Furthermore, Baybutt and Holsber (1990) demonstrated that cortisol reduced differentiation of macrophages and LPS-stimulated IL-1 β production by a macrophage-like human cell line. However, macrophage differentiation and IL-1 β production resumed once cortisol was removed from the culture media. Therefore, the suppression by glucocorticoids is not long-lived and is reversible following the termination of exposure. Glucocorticoids can also modulate the expression of cytokines and their receptors, including pro-inflammatory TNF- α (Ashwell et al., 2000). Corticosteroids have been noted to decrease the secretion of IL-1 β , IL-2,

IL-6 and IFN- γ and increase the receptors for IFN- γ , IL-1 β , IL-6, VP, CRH, serotonin, and insulin (Black, 2002), which will be discussed in more detail later in this section.

Glucocorticoids regulate cytokines, and thus the activation and differentiation of immune cells, through binding to the GR. Specifically, the GR can interfere with the transcription factor activating protein-1 (AP-1; Ashwell et al., 2000). Additionally, the GR increases I κ B, the inhibitor of the transcription factor NF- κ B (nuclear factor- κ B; Ashwell et al., 2000). Both AP-1 and NF- κ B are responsible for increasing the expression of cytokines. Through interfering with gene expression, the GR has been known to inhibit IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, granulocyte macrophage colony stimulating factor (GM-CSF), TNF- α , and IFN- γ (Ashwell et al., 2000). The GR is also responsible for decreasing the stability and half-life of mRNAs for many of these cytokines. In contrast, the activation of the GR has been demonstrated to increase the receptors for IL-1 β , IL-6, GM-CSF, and IFN- γ , and increase cytokine receptor antagonists (Ashwell et al., 2000).

Immune cells other than lymphocytes are also affected by glucocorticoids. For example, administration of dexamethasone to Holstein steers induced neutrophilia, or an increase in circulating neutrophils, while reducing the expression of L-selectin, an adhesion molecule that allows neutrophils to extravasate into tissues (Weber et al., 2004). Glucocorticoids also increase the half-life of neutrophils. This can result in excessive damage to healthy tissue due to their longer life and the extended time in which neutrophils can produce cytotoxic granules (McEwen et al., 1997). Interestingly, neutrophils can release specific proteases that cleave glucocorticoids from cortisol

binding globulin, therefore increasing the amount of “free” cortisol within tissues (McEwen et al., 1997). There is also evidence that stress hormones can affect the maintenance of the memory cell pool (Burns et al., 2003).

In response to infections there is cross-talk between the brain and the immune system. Norepinephrine released from sympathetic neurons (innervating primary and secondary lymphoid organs) and epinephrine, secreted from the adrenal medulla, participate in this cross-talk (Elenkov, 2008). Nearly every immune system organ is innervated by the autonomic nervous system (Williams et al., 1981; Felten et al., 1984; McEwen, et al., 1997). Catecholamine receptors are present on the plasma membrane of lymphocytes as well as other immune cells (Benschop et al., 1996). Additionally, macrophages synthesize and secrete catecholamines in response to activation by LPS, demonstrating that immune cells secrete and are responsive to the effects of catecholamines. Brown et al. (2003) demonstrated macrophages were able to synthesize and secrete norepinephrine 48-hr post-LPS stimulation using a macrophage cell line. The rate limiting enzyme in catecholamine synthesis, tyrosine hydroxylase, was also increased 24 to 48 hr after LPS stimulation, further clarifying that macrophages are indeed synthesizing and secreting catecholamines. Catecholamines administered for an extended period of time (i.e., chronically) decrease the number of circulating lymphocytes and natural killer cells (Livnat et al., 1987). Additionally, catecholamines have effects similar to glucocorticoids in that they suppress the T_H1 type CMI response and promote the T_H2 humoral immune response of the immune system, mainly through regulating cytokine production (Szabó et al., 1997; Hasko et al., 1998; Elenkov, 2008).

However, the secretion of epinephrine in response to a stressor has been suggested to increase the production of IL-6 (Black, 2002). Additionally, β -adrenergic agonists can stimulate the production of IL-8 by macrophages. Therefore, further research is necessary in order to more clearly understand the influence of catecholamines on immune responses.

Other factors can influence the effects of stress hormones on immune function. In particular, immune cells and accessory cells have been reported to express receptors for insulin, prolactin, growth hormone, acetylcholine, endorphins, enkephalins, substance P, somatostatin, estradiol, and testosterone; yet, the number of receptors present depends on the type of immune cell (Besedovsky and del Rey, 1996). Additionally, some of these factors may be secreted by immune cells (Besedovsky and del Rey, 1996). Recent studies have indicated that reproductive hormones can influence the production of various parameters including cytokines, nitric oxide, and stress hormones (Rhodes and Rubin, 1999; Brown et al., 2008). In rats, gonadectomy prevented sound stress-induced decreases in neutrophil phagocytosis in female rats, but not male rats (Brown et al., 2008). Additionally, an increase in estrogen and progesterone during pregnancy is correlated with the shift from T_H1 cytokines to a T_H2 cytokine phenotype, which decreases the ability of the body to defend against intracellular pathogens (Jones et al., 2008). This suggests that progesterone may act to prevent the production of pro-inflammatory cytokines. Furthermore, studies have indicated that the production of stress hormones, in particular cortisol, is sexually dimorphic in that females produce greater concentrations than males (Rhodes and Rubin,

1999). This may be due to the presence of an estrogen response element in the promoter region of the gene for CRH (Tsigos and Chrousos, 2002). In summary, other hormones, in particular reproductive hormones, may influence stress hormone and immune system interactions.

Lymphocytes are also able to produce and respond to stress hormones directly, a concept that was originally posed thirty years ago (Blalock and Smith, 1980; Smith et al., 1982; Besedovsky and del Rey, 1996; Sapolsky et al., 2000). In a study by Smith et al. (1983), Newcastle Disease Virus was injected into female hypophysectomized mice resulting in increases in both corticosterone and IFN- γ . From this study the authors concluded that lymphocytes produced an ACTH-like substance which stimulated the production of corticosterone from the adrenal in response to viral infection.

Additionally, mast cells, involved in the response to allergy, are known to secrete CRH (Elenkov, 2008). Since the study by Blalock and Smith (1980), research has identified several molecules that are released by immune cells including histamine, serotonin, endorphins, insulin, thyroid hormones, oxytocin, gonatotropins, ACTH, vasoactive intestinal peptide, dopamine, and catecholamines (as discussed earlier; Pállinger and Csaba, 2007).

Positive Effects of Stress on Immune Function

It was originally thought that glucocorticoids enhanced defense mechanisms, rather than suppressed them, which came to light with the finding of the anti-inflammatory effects of glucocorticoids in the 1940s (Hench et al., 1949; Munck et al.,

1984). Since that time a vast array of literature has demonstrated the negative effects of stress hormones, particularly chronic stress and the role of glucocorticoids, on immune function. However, more recent literature implies that acute stress can be beneficial, demonstrating that the original hypothesis of the early glucocorticoid physiologists was not completely incorrect. Even so, the negative effect of glucocorticoids on immune function may be beneficial in some ways as it helps to eliminate potentially chronically activated immune cells, which may result in the development of auto-immune reactive cells (Sapolsky et al., 2000). Nevertheless, it is not beneficial to down-regulate the immune system completely in response to a stressor, as the immune system of an animal could potentially be challenged by a pathogen following resolution of the stress response.

Stress hormones, particularly glucocorticoids produced by the adrenal gland, are necessary for the development of the thymus, as adrenal insufficiency or adrenalectomy results in hypertrophy of the thymus that is not reversed by epinephrine administration (Ashwell et al., 2000). Additionally, the ability to respond to glucocorticoids through the GR is necessary for the development of T cells in the thymus, as inhibition of the GR limits the production of mature T cells (Bellinger et al., 2008). Stress hormones are responsible for increases in cholesterol, lipoproteins, triglycerides and free fatty acids, which may be beneficial during the stages of early infections as lipoproteins can bind and thereby neutralize LPS (Black, 2002).

Studies have also demonstrated that cortisol administered 144 hr or less prior to an endotoxin (LPS) challenge can enhance the cytokine response, perhaps by priming

the immune system (Besedovsky et al., 1996; Sapolsky et al., 2000; Sorrells and Sapolsky, 2007). In contrast, glucocorticoid administered at the same time or after the administration of endotoxin suppresses these responses (Sapolsky et al., 2000). Additionally, in a review by Sorrells and Sapolsky (2007), the authors stated that the early response to invading pathogens is characterized by low concentrations of glucocorticoids that are permissive; later responses to pathogens, characterized by high concentrations of glucocorticoids, results in the negative effects on immune function. Therefore, the timing relative to immune system activation is extremely important in determining whether stress hormones will have stimulatory and inhibitory effects on the immune system.

The products of the stress response (i.e. glucocorticoids and catecholamines) alone, in the absence of an infection, can activate the immune system. For example, the immune system is known to be activated in response to physical trauma, psychological stressors, radiation, oxidative stress, ischemia, and extreme temperatures. In response to these stressors, activation of the immune system sometimes occurs prior to stressor-induced increases in stress hormones (Sapolsky et al., 2000; Linde et al., 2008). Additionally, the production of IL-1 β can stimulate the production of both CRH and ACTH from the hypothalamus and pituitary, respectively (Sapolsky et al., 2000).

Similar to glucocorticoids, catecholamines have also been labeled as primarily immunosuppressive; yet new literature suggests that this is not completely true (Elenkov, 2008). Acutely, catecholamines can increase the number of innate immune cells, particularly NK cells (Elenkov, 2008). Additionally, acute stress activation of the

immune response is mediated by the sympathetic nervous system, including enhanced neutrophil proliferation and survival (Sorrells and Sapolsky, 2007). However, there are limited studies describing positive effects of catecholamines on the immune system, as previous studies have primarily focused on the effects of glucocorticoids.

Most if not all of the studies discussed previously utilized rodent models to elucidate positive mechanisms of action on the immune system. These models may not be appropriate for humans and livestock species such as cattle due to the use of inbred and genetic selection in mice strains. Additional studies are warranted in larger livestock species in order to determine if similar immune system stimulation and permissive actions occur in response to acute glucocorticoid stimulation.

In summary, stress hormones can have both inhibitory and stimulatory effects on immune function. As different stressors can elicit different physiological and endocrinological responses, the following two sections will review literature pertaining specifically to transportation and inflammatory stressors.

Transportation of Cattle

Transportation is a common and necessary management procedure in the livestock industry. This is especially true for the cattle industry, which often requires multiple transportation events throughout the production cycle. Many early studies focused on the combined stress of weaning and then transportation. Crookshank et al., (1979) demonstrated that weaning and transportation of calves increased cortisol concentrations one day after the events which then decreased to baseline values between

4 and 7 d post weaning and transportation. Furthermore, cattle that were handled multiple times had lower concentrations of cortisol, demonstrating that calves have the ability to acclimate to handling. Similar suggestions have been made in regards to improvement of temperament with constant handling.

In the early 1980s, the focus began to shift to the study of the effects of transportation on immune function. Murata et al. (1985) transported Holstein calves for 1 hr and found no increase in cortisol concentration 6 hr post-transportation; rather cortisol concentrations were lower than pre-transportation values. In addition, proliferation by isolated lymphocytes was increased post-transportation, along with the activity of isolated neutrophils. However, the samples collected at 6 hr were the first samples collected immediately following transportation (i.e., no samples were collected following the cessation of transportation). It is quite possible that the authors would have found different results had samples been collected immediately after the end of transportation. Indeed, in a study conducted 2 years later utilizing Holstein calves, Murata et al. (1987) found an increase in cortisol concentrations collected immediately following the end of a 4-hr transportation. In addition, the authors demonstrated that transportation increased leukocytosis, mainly due to an increase in circulating neutrophils (neutrophilia). However, there was a non-significant decrease in total lymphocytes and eosinophils. Moreover, the number of T cells was lower post-transportation and remained lower for up to 20 hr, yet B cell numbers were unaffected. This probably accounted for the decrease in basal lymphocyte proliferation, which is in

contrast to their previous study in which they found an increase in lymphocyte proliferation.

Around the same time Blecha et al. (1984) transported Brahman and Brahman-Angus cross steers 10 hr and found an increase in leukocytes (due to neutrophilia) at unloading which persisted 7 d following transportation. Proliferation of lymphocytes isolated and cultured after unloading was lower compared to non-transported controls. The authors suggested this may be due to the negative affect of cortisol on the cytokine responsible for lymphocyte proliferation, IL-2; however, they found no difference in cortisol concentrations at the end of transportation when compared with non-transported controls. The authors suggested cortisol concentrations may have increased during the transportation period when samples were not collected, and decreased prior to the collection of the sample following the end of transportation.

In more recent studies, Arthington et al. (2003), using Brahman crossbred calves, did not see an increase in cortisol concentrations following a 3-hr transport. However, Buckham Sporer et al. (2007) found an increase in cortisol concentrations 4.5 hours into a 9.75 hour transportation of Belgian Blue x Friesian bulls. In addition, neutrophilia was present at 4.5 hours and peaked at the end of transportation, which tended to be weakly correlated with cortisol concentrations ($r = 0.33$; $P = 0.07$). The authors also found changes in gene expression of neutrophil genes responsible for regulating inflammation and gram negative bacteria clearance. In a comparable study published in 2008, Buckham Sporer et al. found a similar increase in neutrophil number and an increase in

total leukocytes in a study utilizing Aberdeen Angus, Friesian, and Belgian Blue-Friesian cross bulls transported for 9 hr.

As noted by the studies described above, there are multiple studies that utilize cortisol as a marker for stress in response to transportation. Contrarily, there are limited studies that discuss changes in catecholamine concentrations in response to transportation. Specifically, norepinephrine, but not epinephrine, decreased in response to a 14-hr transport of six-month old Blonde d'Aquitaine calves (Odore et al., 2004). Additionally, both the lymphocyte protein concentrations of the GR and the β -adrenergic receptors were decreased following transportation (Odore et al., 2004).

In summary, transportation clearly affects the number of circulating leukocytes, mainly circulating neutrophils. The aforementioned studies also found a decrease in lymphocyte proliferation in response to transportation. Although both leukocytosis and lymphocyte proliferation are often attributed to the effects of cortisol, none of the authors acknowledged the possible role of catecholamines. Indeed, catecholamine concentrations were not determined in response to transportation in any of these previous studies. Additionally, these previous studies did not account for the possible influence of temperament on cortisol concentrations. Furthermore, there is inconsistency in the results in regards to the production of cortisol in response to transportation. However, it is possible that this is due to differences in breed, animal temperament, and in the duration of transportation. Therefore, further studies are warranted to elucidate if transportation induces changes in epinephrine as well as

norepinephrine and cortisol, and to determine if these responses are influenced by temperament of cattle.

Inflammatory Responses Induced by Endotoxin

Inflammatory stress, or stress induced by inflammatory stimuli, can greatly affect how an animal responds to and recovers from an infection. While inflammation is a response of the innate immune system and is essential for early immune actions, it also plays an important role in activating the adaptive immune system (Männel, 2007). A common antigen used to mimic an animal's response to gram negative bacterial infections is lipopolysaccharide (LPS; endotoxin; Männel, 2007). Lipopolysaccharide is a component of the cell wall of gram negative bacteria, such as *E. coli*. The cell wall of gram negative bacteria has 2 layers: a inner thin peptidoglycan layer and an outer asymmetric lipid bilayer interspersed with proteins (Beveridge, 2001). The outer layer is almost exclusively composed of LPS, which is critical for membrane stability (Leon et al., 2008). In contrast, gram positive bacteria have a multi-layered cross-linked polymer called peptidoglycan surrounding the plasma membrane (Leon et al., 2008). The biologically active component of LPS, Lipid-A, is recognized by TLR-4 after binding to a serum protein, LBP, MD-2 and CD-14 (Storni et al., 2005; Männel, 2007; Leon et al., 2008).

In response to LPS (endotoxin), cells of the immune system will produce pro-inflammatory cytokines, primarily TNF- α , IL-1 β , and IL-6. These cytokines initiate the inflammatory response and can also stimulate the production of other inflammatory

mediators. However, inflammatory mediators produced in excessive amounts can lead to multiple organ failure possibly resulting in death (Avitsur et al., 2006). In order to prevent a hyper-inflammatory state, the stress response is also stimulated, primarily by the production of pro-inflammatory cytokines within the brain (Rhen and Cidlowski, 2005). Specifically, pro-inflammatory cytokines produced locally in the brain, and secreted systemically in circulation, can stimulate the secretion of CRH, ACTH, and cortisol from the hypothalamus, anterior pituitary, and adrenal gland, respectively. The production of stress hormones is essential for an appropriate response to LPS. For example, the administration of *E. coli* to adrenalectomized rodents resulted in a 100-fold greater lethal sensitivity compared to sham-operated controls, a response which was not seen when dexamethasone was administered at the same time as *E. coli* (Sapolsky et al., 2000; Silverstein and Johnson, 2003). However, studies have indicated that various stressors can influence the ability of an animal to produce stress hormones, particularly glucocorticoids, in response to endotoxin challenge. For example, mice subjected to social defeat stress prior to endotoxin challenge are more likely to die due to glucocorticoid resistance stimulated by the elevated glucocorticoids associated with the social defeat stress (Avitsur et al., 2006).

Lipopolysaccharide, bound to TLR-4 on cells of the innate immune system, activates signal transduction mechanisms resulting in production of pro-inflammatory cytokines and chemokines (Hoshino et al., 1999; Underhill and Ozinsky, 2002; Männel, 2007). The central mediator of the effects of LPS is the secretion of TNF- α . It is detected in the serum of several animal species in response to LPS, and the infusion of

TNF- α can induce a shock-like state (Bieniek et al., 1998). Additionally, LPS induces the secretion of IFN- γ (Bieniek et al., 1998). Stress hormones also increase in response to LPS injection. For example in pigs, Williams et al. (2007) demonstrated that cortisol, epinephrine, and norepinephrine all increased in response to LPS challenge, suggesting that stress hormones may have a role in modulating the production of pro-inflammatory cytokines post-LPS challenge. Cytokines stimulate the production of epinephrine and norepinephrine early in an inflammatory response (van der Poll, 2000). Similarly, catecholamines can modulate the production of cytokines in the later stages of an inflammatory response. Catecholamines can act directly or indirectly with LPS to increase the production of cytokines from macrophages, synergistically with LPS-stimulated corticosteroid induction of IL-6 and TNF- α production (Black, 2002). It is hypothesized that a similar temporal endocrine response to LPS occurs in cattle.

In response to inflammation, glucocorticoids such as cortisol inhibit the recruitment, proliferation, survival, and degranulation of mast cells and therefore limit the inflammatory response (Chapman et al., 2009). Benshop et al. (1996) demonstrated that catecholamines increase in response to infection and sepsis in both human and animal models. Kizaki et al. (2008) demonstrated that while LPS down-regulates the β_2 -adrenergic receptor, overexpression of the receptor prevents TLR-4 signaling. Furthermore, studies indicate that β_2 -adrenergic receptors are involved in inhibiting pro-inflammatory cytokine production in response to LPS and α -adrenergic receptor activation augments cytokine production (Ignatowski et al., 1996). Signal transduction initiated by LPS binding to TLR-4 can lead to adaptive responses through induction of

maturation of dendritic cells and by influencing T cell responses through the production of various cytokines (Männel, 2007). Therefore, stress hormones can modulate the immune response to LPS through alterations in cell surface receptors and the production of cytokines.

Following up-regulation of the innate and later the adaptive immune system, the body must resolve the diseased state and return to homeostasis. The failure or decreased ability of an immune response to remove a pathogen, due to 1) a general paralysis of the immune system or 2) excessive inflammation from the over-production of inflammatory mediators, results in the condition septic shock (Pfeffer, 2003; Leon et al., 2008). The ultimate resolution of inflammation is influenced by both innate immune cells and stress hormones, in particular cortisol. There are several steps that must be accomplished in order to resolve the diseased state. The initial signal is a decrease in the number of microbes in the inflamed tissue. As the number of microbes decrease, macrophages begin secreting anti-inflammatory cytokines that suppress inflammation, including transforming growth factor- β (TGF- β ; Serhan and Savill, 2005). This cytokine acts to inhibit signaling through TLRs that promote inflammation, and stimulates the consumption of key mediators of inflammation (Männel, 2007). The production of TGF- β also stimulates the differentiation of another subtype of T cells, T-regulatory cells. T-regulatory cells stimulate reduction and resolution of inflammation (Männel, 2007). Furthermore, macrophages and neutrophils are stimulated to undergo apoptosis, limiting possible damage to healthy tissue and promoting tissue repair (Männel, 2007). It is believed that cortisol plays a role in the activity of macrophages in inflamed tissue

through the actions of HSD11 β type 1 (Serhan and Savill, 2005). This enzyme is responsible for converting cortisone to cortisol and is expressed by macrophages. Glucocorticoids also have a role in promoting apoptosis of eosinophils and dying leukocytes (Serhan and Savill, 2005). Additionally, glucocorticoids and catecholamines increase the production of LBP by the liver; therefore, neutralizing and limiting the toxicity of LPS (Black, 2002). Kupffer cells, a specialized liver macrophage, as well as pulmonary intravascular macrophages in the lung, are important in the clearance of LPS from circulation (Chitko-McKown et al., 1992; Black, 2002).

Sickness Behavior. In response to cytokine stimulation of the hepatic vagal afferent nerves, a characteristic pattern of behavior will occur, described as sickness behavior (Black, 2002). This behavioral response can also be stimulated through the actions of cytokines within the brain (Borderas et al., 2008). Sickness behavior is a sum of behavior changes characterized by reduced feed intake, reduced motion or movement, reduced social interaction, reduced sexual activity, and increased sleep (Black, 2002; Borderas et al., 2008). While there is a large amount of literature on LPS-induced sickness behavior in rodent models, there are few published reports on the sickness behavior response of cattle to an endotoxin challenge. A study using low doses of LPS (0.025 and 0.05 $\mu\text{g/kg BW}$) in dairy calves found that LPS reduced the duration of rumination, time spent eating hay, and frequency of grooming, but increased the frequency of calves standing inactive and the total duration of time lying inactive (Borderas et al., 2008). Understanding the sickness behaviors elicited in response to a

certain illness will help develop or change management practices in order to treat animals more efficiently.

TEMPERAMENT AND IMMUNE FUNCTION

Recent studies have indicated that temperament negatively impacts immune function. Cavigelli et al. (2007) indicated that mice showing high-locomotion behavior in response to novel stimuli have larger adrenals, greater concentrations of corticosterone, and lower concentrations of TNF- α after tail-nicking. These mice also had a greater incidence of tumor formation and often died at a younger age than less-responsive mice. Therefore, high-responsive mice have a greater HPA axis activation with a coincident hampered immune response.

Along with having negative impacts on growth and carcass quality, temperament can negatively affect immune function of cattle. In a study conducted in Brahman steers, Temperamental steers had lower *in vitro* lymphocyte proliferation and lower *in vivo* vaccine-specific IgG concentrations when compared to Calm steers (Oliphint et al., 2006). However, there is limited documentation of the influence of temperament on immune function in cattle. Specifically, the influence of temperament on the production of stress hormones in response to an immune challenge has yet to be studied in sufficient detail.

Studies using chronic stressors in mice have found changes in immune function. For example, mice exposed to social defeat stress multiple times have been found to have developed glucocorticoid resistance (as discussed previously), increasing the

probability of mice dying from endotoxic shock (Avitsur et al., 2006). Additionally, elevated glucocorticoids have been demonstrated to impair clearance of bacteria from wounds and wound healing. However, glucocorticoid resistance may be beneficial in mice that are more aggressive, as this allows wounds to heal more quickly in the presence of greater concentrations of glucocorticoids. In cattle it remains unclear whether the greater basal concentrations of glucocorticoids and catecholamines, characteristic of temperamental cattle, is immunosuppressive, or whether, in response to specific challenges, stress hormones can be beneficial.

SUMMARY AND OBJECTIVES

In summary, these findings suggest that there is a link between stress, temperament, and immunity. However, the relationships may depend on the type of stressor and the duration of the stress (chronic versus acute). We hypothesize that temperament will affect the stress response (temporal pattern of cortisol and epinephrine release) to transportation and LPS-induced inflammation. Due to interactions of the stress response and temperament, innate immunity, the cell mediated response (lymphocyte proliferation), and the humoral response (antibody production) may be lower in more temperamental calves. Additionally, we hypothesize that acute increases in cortisol, manifested in response to acute stress or stimulation of endogenous cortisol secretion by ACTH, may positively influence immune function and prepare cattle for subsequent immune challenge.

Based on these findings we set the following objectives:

1. To determine the influence of temperament on stress hormones, physiological measurements of rectal temperature and heart rate, and proliferation, IgM production and gene expression of isolated peripheral blood mononuclear cells in response to transportation;
2. To determine the influence of temperament on the rectal temperature and stress hormone responses to LPS challenge;
3. To determine the effects of acute increases in endogenous cortisol on gene expression in peripheral blood mononuclear cells.

CHAPTER III

RELATIONSHIPS BETWEEN TEMPERAMENT AND TRANSPORTATION WITH RECTAL TEMPERATURE AND SECRETION OF CORTISOL AND EPINEPHRINE IN BULLS¹

INTRODUCTION

Livestock production practices (e.g., ear tagging, branding, castration, and vaccination), social mixing, and transportation have been reported to be stressful and induce secretion of the stress hormones cortisol, epinephrine, and norepinephrine (Crookshank et al., 1979; Rulofson et al., 1988; Lay et al., 1992; Carrasco and Van de Kar, 2003; Charmandari et al., 2005; Buckham Sporer et al., 2008). Acute stress is not necessarily detrimental to the health of an animal, and may even be beneficial (Galyean et al., 1999; Dhabhar, 2002; Duff and Galyean, 2007; Sorrells and Sapolsky, 2007). However, chronic stress can negatively impact growth, reproductive function, and immune function (Moberg, 1987; Dobson et al., 2001; Shi et al., 2003; Silberman et al., 2003; Compas et al., 2004; Zhao et al., 2008). Therefore, minimizing multiple stressful incidents may be beneficial to health and growth of livestock.

The effect of animal temperament on health and performance is an area of increasing research interest. In cattle temperament is defined as the reactivity, or fear response, to humans (Fordyce et al., 1988). Temperament has been correlated with

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concentrations of stress hormones in cattle, in that more temperamental, or excitable, cattle have greater concentrations of cortisol and epinephrine (Schuehle et al., 2005; King et al., 2006; Curley et al. 2006a,b, 2008). Analogous to stress, temperament can have negative impacts on growth (average daily gain), carcass traits and immune function in cattle with poor temperaments (Voisinet et al., 1997; Fell et al., 1999; Mondal et al., 2006; Oliphint et al., 2006).

Transportation has been purported as a stressor in the livestock industry, yet interestingly there have been limited studies in cattle that have demonstrated increases in rectal temperature. Tarrant et al. (1992) did not find a change in rectal temperature measured before and after a 24-hr transport of Friesian steers. In addition, a shorter 9-hr transport of young beef bulls did not find a transport-induced difference in rectal temperature, measured by using a hand-held digital thermometer (Buckham Sporer et al., 2008). Yet, rectal temperature of their bulls was lower 48 hr after the initiation of transportation. In contrast, rectal temperature increased in heifers that were transported for 4 hr on two consecutive days compared to non-transported controls (Behrends et al., 2009). However, limited information is available on the effect of transportation on changes in rectal temperature in cattle which do not have the influence of human presence during the data collection.

Therefore, our study was designed to determine the influence of temperament on rectal temperature recorded without human presence and secretion of cortisol and epinephrine in bulls in response to transportation.

MATERIALS AND METHODS

Experimental Design

Bulls (10 mo of age) from the Texas AgriLife Research Center's purebred Brahman herd in Overton, TX were selected for use in this study based on their temperament score measured 28 days prior to weaning (133 ± 3 days of age). Temperament score (Curley et al., 2006a; King et al., 2006) was an average of exit velocity (EV) and pen score (PS). Exit velocity is an objective measurement that records the rate (m/s) at which cattle exit a working chute (Burrow et al., 1988; Curley et al., 2006a). Pen score (Hammond et al., 1996) is a subjective measurement in which cattle are separated into small groups of three to five and their reactivity to a human observer scored on a scale of 1 (calm, docile, approachable) to 5 (aggressive, volatile, crazy). Based on temperament score the 8 most Calm (0.89 ± 0.15 EV and 1.00 ± 0.00 PS), 8 most Temperamental (3.70 ± 0.29 EV and 4.88 ± 0.13 PS), and the 8 Intermediate bulls (1.59 ± 0.12 EV and 2.25 ± 0.16 PS) were selected from a pool of 60 bulls (Fig. 1). Prior to transportation bulls were fitted with rectal temperature recording devices (A HOBO Pro v2 Temp data logger probe; Part # U23-004, Onset Corp., Pocasset, MA) that measured rectal temperature continuously at 1-min intervals in the absence of a human operator. The factory-calibrated rectal temperature recording devices were tested for accuracy upon receipt from the manufacturer. However, several rectal temperature recorders became displaced during transportation. Specifically, temperature data presented includes only those bulls that yielded a complete data set ($n = 5, 8,$ and 4 for Calm, Intermediate and Temperamental, respectively). Once all bulls were loaded on

the trailer continuous recording of the data from the rectal temperature devices commenced (i.e., time 0, initiation of transportation). Prior to and after transportation, while bulls were restrained in a working chute, whole blood samples (2 x 10 mL) were collected via jugular venipuncture in uncoated or EDTA coated tubes (BD, Franklin Lakes, NJ) and serum and plasma isolated for determination of cortisol and epinephrine concentrations, respectively. Bulls were loaded into an 11-m open-sided livestock trailer and transported approximately 770 km (9 hr) from Overton, TX (32.27 N, -94.98 W, 153 m altitude) in East Texas to New Deal, TX (33.74 N, -101.84 W, 1006 m altitude) in the South Plains of West Texas. This study took place on November 11th, 2007 when the average ambient temperatures were 15°C and 10°C for Overton and New Deal, respectively. All experimental procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committees of Texas A&M University and the USDA.

Cortisol

Serum concentrations of cortisol were determined using a single antibody radioimmunoassay (DSL-2100; Diagnostic Systems Labs, Webster, TX) utilizing rabbit anti-cortisol antiserum coated tubes according to the manufacturer's directions (Burdick et al., 2009). The minimum detectable cortisol concentration was 1.2 ng/mL and the intra- and inter-assay coefficients of variation were 4.3% and 2.4%, respectively. Serum

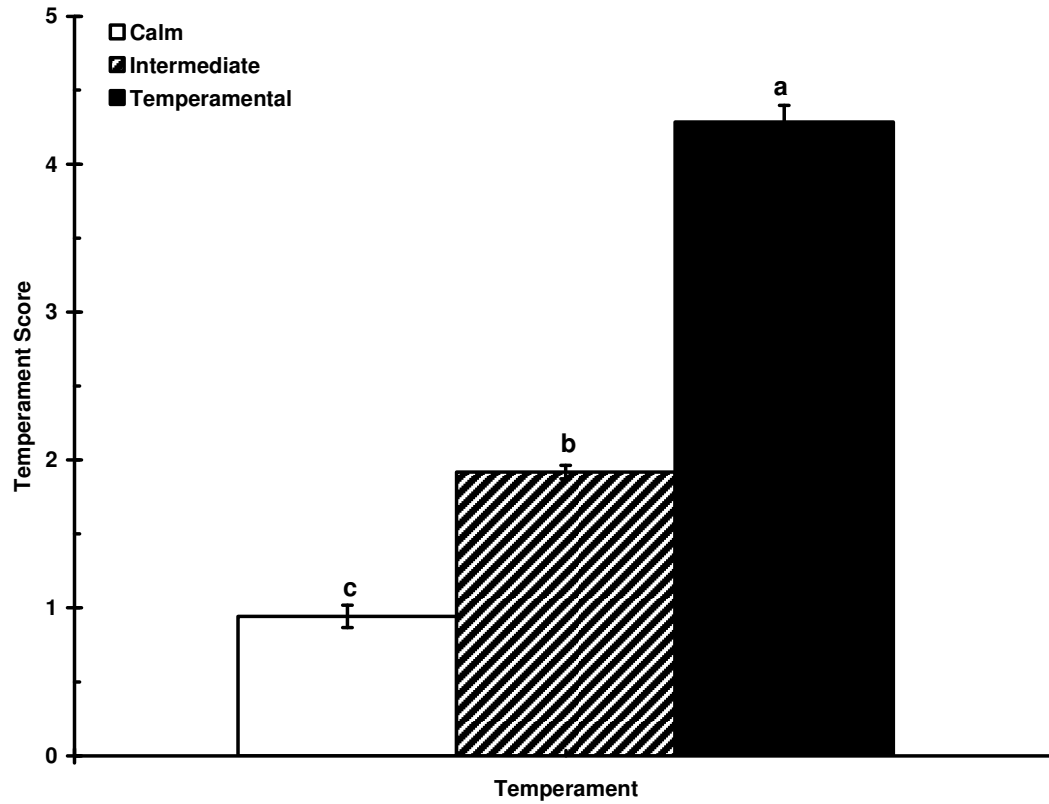


Figure 1. Temperament score of Purebred Brahman bulls (10 mo of age) selected based on temperament score measured 28 d prior to weaning. Temperament score was an average of exit velocity (EV) and pen score (PS). Means with unlike letters differ by $P < 0.05$.

concentrations of cortisol were determined by comparison to a standard curve generated with known concentrations of cortisol and presented as the concentration in ng/mL

Epinephrine

Plasma concentrations of epinephrine were determined by enzyme immunoassay according to the manufacturer's directions (17-BCTHU-E02; Alpco Diagnostics, Boston, MA; Burdick et al., 2009) by comparison of unknowns to standard curves generated with known concentrations of epinephrine. Data are presented as pg/mL. The minimum detectable epinephrine concentration was 11 pg/mL and the intra- and inter-assay coefficients of variation were 3.7% and 7.4% respectively.

Statistical Analysis

Data for rectal temperature, cortisol, and epinephrine were analyzed using ANOVA specific for repeated measures (Statview, SAS Inst., Inc., Cary, NC). Sources of variation included temperament, time and their interactions. Specific treatment comparisons were made using Fisher's Protected Least Significant Difference, with $P < 0.05$ considered significant. Pearson's correlation coefficients were also calculated for the following variables: minimum and maximum rectal temperature, EV, pre- and post-transportation cortisol and epinephrine concentrations (Statview, SAS Inst., Inc., Cary, NC).

RESULTS

Rectal Temperature

Prior to transportation (Fig. 2; 0 min) Temperamental bulls had greater rectal temperatures than Calm and Intermediate bulls ($P < 0.05$). After the onset of transportation, maximal rectal temperatures was reached within 30 min, with Temperamental bulls having greater maximal rectal temperatures than Calm and Intermediate bulls (Fig. 3; $P < 0.05$). After reaching maximum values, rectal temperatures decreased through 400 min from the onset of transportation. At that time Calm bulls had lower mean rectal temperatures than Intermediate and Temperamental bulls (Fig. 4; $P < 0.05$). During transportation, the truck made refueling stops at 3 time points: 30-50 min, 200-220 min, and 360-380 min (Fig. 2). During or immediately after the three stops rectal temperatures decreased slightly in all bulls before returning to pre-stop values.

Cortisol and Epinephrine

Prior to transportation, Temperamental bulls had greater concentrations of cortisol than Calm bulls ($P < 0.05$; Fig. 5). Similarly, upon arrival Temperamental bulls had greater cortisol concentrations than Calm bulls. Pre- and post-transportation cortisol concentrations did not differ between Calm and Intermediate bulls. However, within Temperamental bulls cortisol concentrations tended to be lower post-transportation compared to pre-transportation ($P = 0.07$).

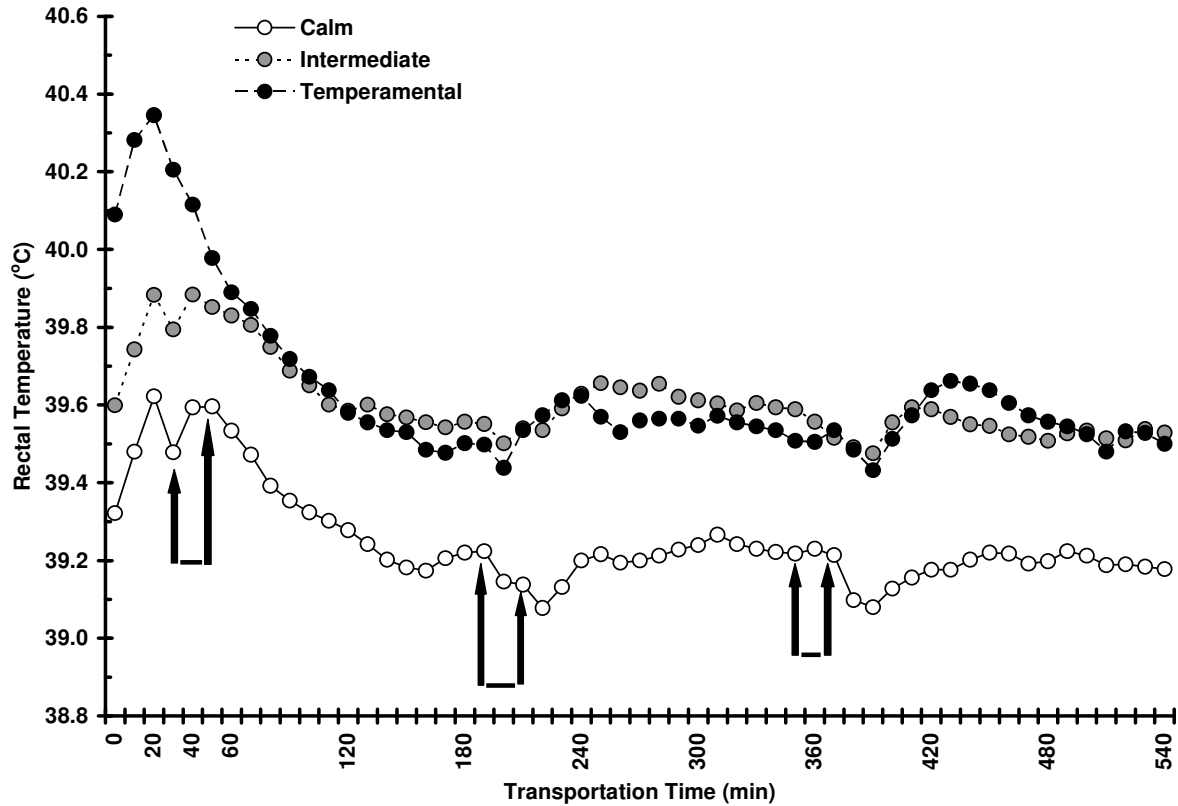


Figure 2. Rectal temperature of Brahman bulls during transportation for 9 hr. Data presented for Calm ($n = 5$; $SEM \pm 0.07$), Intermediate ($n = 8$; $SEM \pm 0.13$) and Temperamental ($n = 4$; $SEM \pm 0.12$) bulls. Arrows represent periods of time in which the truck and trailer were stopped for refueling. Prior to transportation Temperamental bulls had greater rectal temperatures than Calm and Intermediate bulls ($P < 0.05$). Maximum rectal temperature was reached within 30 min of the onset of transportation, with Temperamental bulls having greater maximum values than Calm and Intermediate bulls ($P < 0.05$). After reaching maximum values, rectal temperatures decreased through 400 minutes from the onset of transportation. At that time Calm bulls had lower mean rectal temperatures than Intermediate and Temperamental bulls ($P < 0.05$).

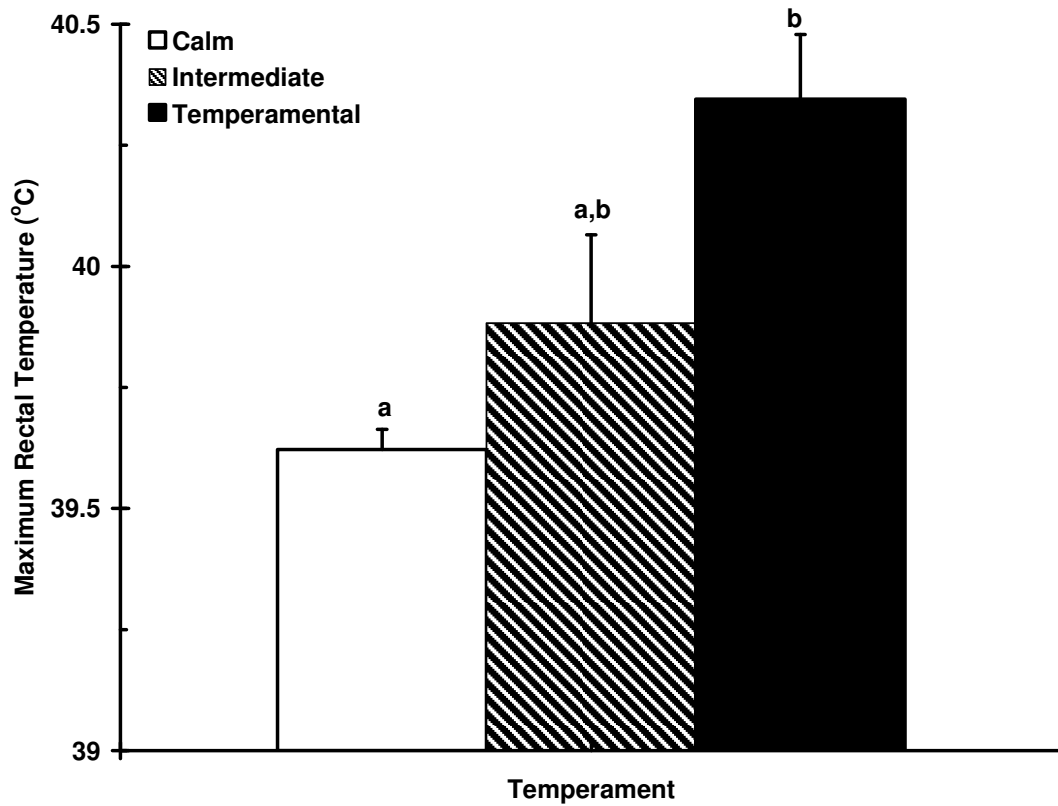


Figure 3. Maximum rectal temperature of Brahman bulls attained during a 9-hr transportation. Maximum rectal temperature was achieved at 30 minutes. Temperamental bulls had greater peak rectal temperature than Calm bulls ($P < 0.05$). Data presented for Calm ($n = 5$; $SEM \pm 0.07$), Intermediate ($n = 8$; $SEM \pm 0.13$) and Temperamental ($n = 4$; $SEM \pm 0.12$) bulls. Means with unlike letters differ by $P < 0.05$.

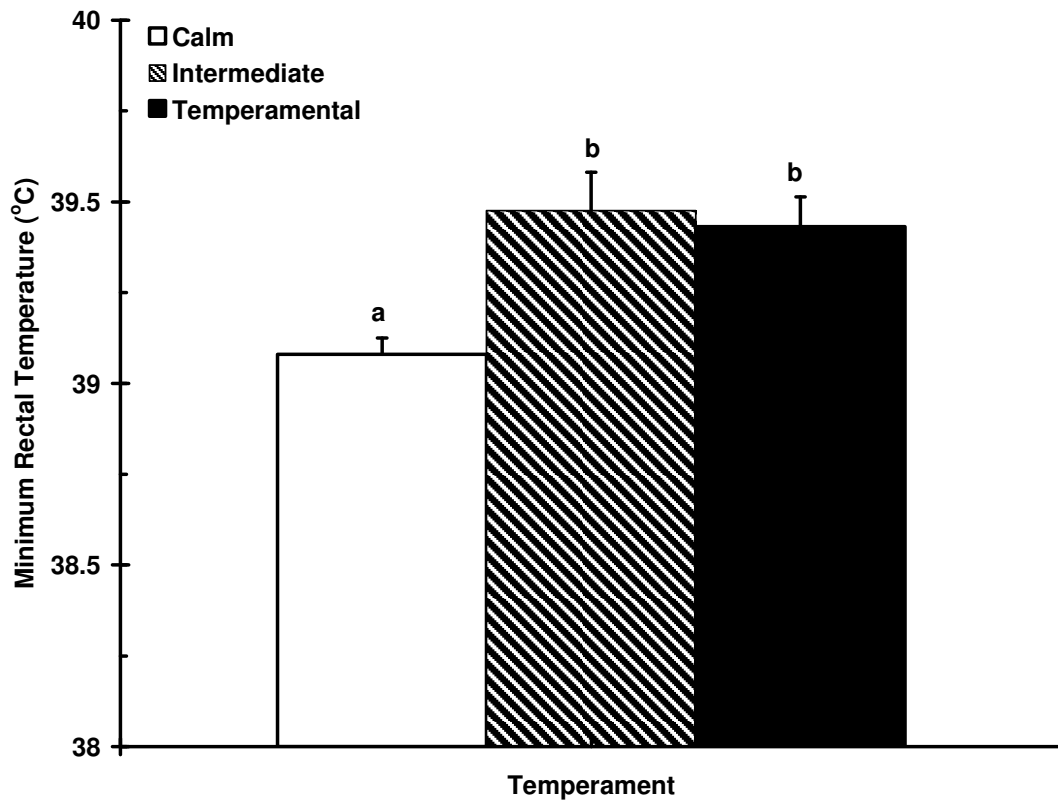


Figure 4. Minimum rectal temperature of Brahman bulls attained during a 9-hr transportation. Minimum rectal temperature was achieved at 400 minutes. Temperamental and Intermediate bulls had greater minimum rectal temperature than Calm bulls ($P < 0.05$). Data presented for Calm ($n = 5$; $SEM \pm 0.07$), Intermediate ($n = 8$; $SEM \pm 0.13$) and Temperamental ($n = 4$; $SEM \pm 0.12$) bulls. Means with unlike letters differ by $P < 0.05$.

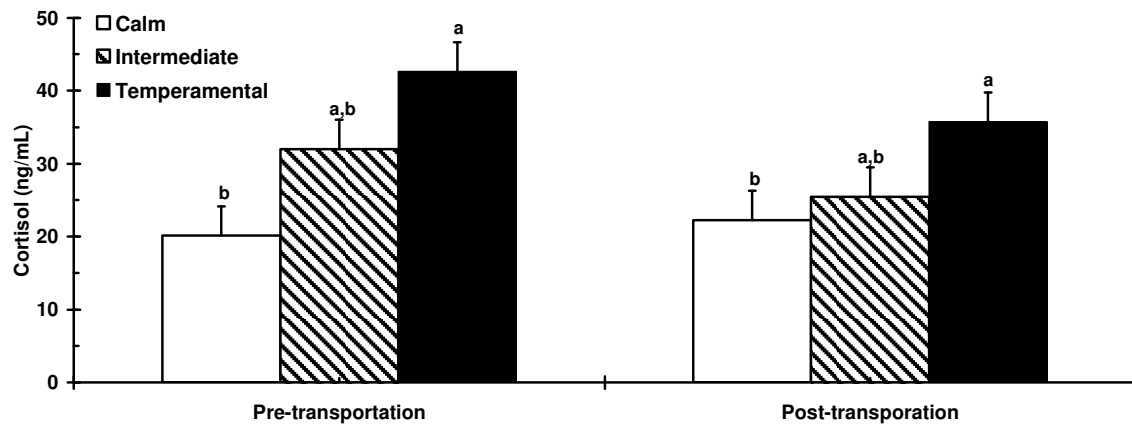


Figure 5. Serum cortisol concentrations of Brahman bulls pre- and post-transportation for 9 hr ($n = 8$ Calm, 8 Intermediate and 8 Temperamental bulls). Pre-transportation cortisol values were not different than post-transportation values ($P > 0.05$). However, Temperamental bulls had greater cortisol concentrations than Calm bulls ($P < 0.05$). Means with unlike letters differ by $P < 0.05$.

Temperamental bulls also had a greater concentrations of epinephrine prior to transportation than Calm or Intermediate bulls ($P < 0.01$; Fig. 6). Likewise, post-transportation Temperamental bulls had greater concentrations of epinephrine than Calm or Intermediate bulls ($P < 0.01$). Within groups, there were no differences in pre- and post-transportation epinephrine concentrations ($P > 0.05$). However, concentrations of cortisol and epinephrine were greater in Temperamental bulls than Calm bulls in agreement with previous publications from our laboratory (Curley et al., 2006a, 2008).

Relationships Amongst Temperament, Rectal Temperatures and Stress Hormones

Maximum rectal temperature was reached 30 min after the onset of transportation, minimum rectal temperature was reached by 400 min after the onset of transportation, and these parameters were positively correlated with each other ($r = 0.73$; $P < 0.01$). There was a positive correlation between EV and maximum rectal temperature ($r = 0.62$; $P = 0.01$) and EV tended to be positively correlated with minimum rectal temperature ($r = 0.43$; $P = 0.10$). Pre-transportation epinephrine concentrations tended to be positively correlated with maximum rectal temperature reached within 30 min after the onset of transportation ($r = 0.46$; $P = 0.06$). In agreement with previously published literature, cortisol (pre-transportation) was positively correlated with EV ($r = 0.55$; $P = 0.02$; Curley et al., 2006a). Likewise, both pre- and post-transportation epinephrine concentrations were positively correlated with EV ($r = 0.64$; $P < 0.01$ and $r = 0.59$; $P < 0.01$, respectively).

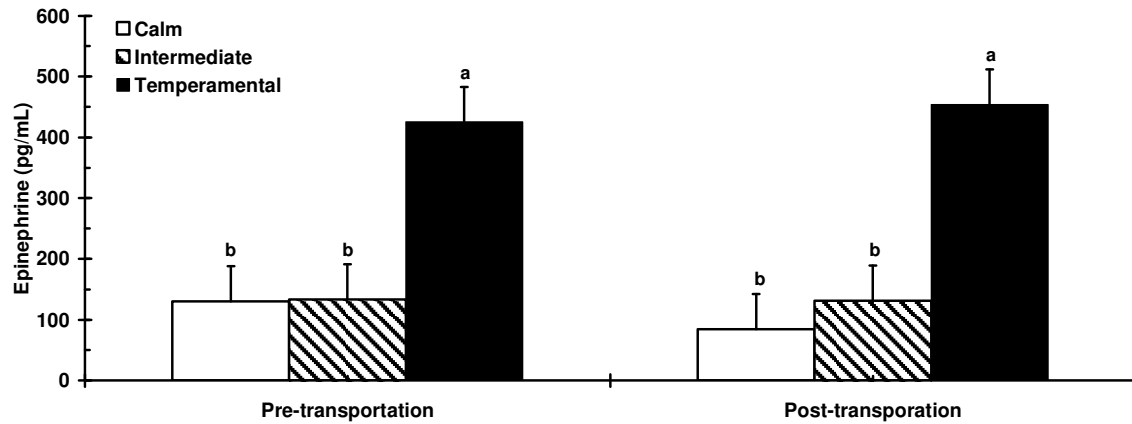


Figure 6. Plasma epinephrine concentrations of Brahman bulls pre- and post-transportation for 9 hr ($n = 8$ Calm, 8 Intermediate and 8 Temperamental bulls). Pre-transportation epinephrine values were not different than post-transportation values ($P > 0.05$). However, Temperamental bulls had greater epinephrine concentrations than Calm and Intermediate bulls ($P < 0.05$). Means with unlike letters differ by $P < 0.05$.

DISCUSSION

This study investigated whether temperament influences rectal temperature measured continuously in the absence of a human operator and the secretion of cortisol and epinephrine in response to transportation of pre-pubertal Brahman bulls. In this study bulls were subjected to 9-hr of transportation. Transportation induced an increase in rectal temperature in all bulls, with rectal temperatures being greater in Temperamental than Calm bulls. Minimal rectal temperature, reached at 400 min, was also greater in Temperamental and Intermediate bulls than Calm bulls. Cortisol and epinephrine concentrations were greater in Temperamental bulls; however, neither cortisol nor epinephrine concentrations differed between pre- and post-transportation time points within groups.

At the initiation of transport rectal temperatures were greater in Temperamental bulls when compared to Calm and Intermediate bulls. This could be explained by either Temperamental bulls having a greater basal rectal temperature than Calm and Intermediate bulls, or that an increase in rectal temperature was stimulated by the initial processing prior to transportation (i.e., blood sampling, rectal temperature device placement, and loading) which was more stressful to Temperamental bulls than Calm and Intermediate bulls. Previous studies in our lab (unpublished data) have not found differences in resting rectal temperature due to temperament, which supports the later explanation. Acute stress has been documented to increase body temperature, a response attributable to the secretion of catecholamines (Oliver et al., 2005). Therefore, if Temperamental bulls were more reactive to the initial handling prior to and during

loading, this would suggest a greater increase in rectal temperature by these bulls compared to Calm and Intermediate bulls.

Rectal temperature displayed an initial increase within the first 30 min before decreasing throughout the remainder of transportation. A study by Tarrant et al. (1992), in which cattle were transported for 24 hr, also found an increase in rectal temperature. In contrast, Buckham Sporer et al. (2008) did not detect differences in rectal temperature before or after transportation of cattle for 9 hr. However, this does not dismiss the possibility that rectal temperature changed during transportation. In the aforementioned study, rectal temperature was determined using a digital thermometer and was only measured at 3 time points (-24, 9.75 and 48 hr) relative to transportation. The current study measured rectal temperature remotely throughout the transportation period thus allowing for determination of continuous temporal changes. By use of rectal temperature recording devices similar to those used in the current study, Behrends et al. (2009) determined that rectal temperature increased in heifers subjected to a 4-hr transport on 2 consecutive days. In the current study rectal temperature did not remain elevated throughout the transportation period, decreasing from its peak 30 min after the initiation of transportation. Behrends et al. (2009) also found a decrease in rectal temperature following the initial increase, suggesting that transportation is only acutely stressful and that the heifers were able to acclimate to transportation. While it is not completely clear as to why rectal temperatures remained low following the decline from peak values, it is possible that the circulating air during transportation was able cool the animals following the acclimation of the animal (decline in rectal temperature from peak

values). Also, rectal temperature may decrease if the stimulus, potentially catecholamines, decreases.

Pre-transportation cortisol concentrations were slightly elevated in Calm and Temperamental bulls compared with previously published baseline cortisol concentrations for Brahman bulls of a similar age (Curley et al., 2006a). Therefore the handling of the bulls in our study prior to and following transportation may have influenced cortisol concentrations and prevented the detection of differences before and after transportation. This suggests that more frequent collection of blood samples (mainly during the transport itself) to determine concentrations of cortisol and epinephrine are necessary to identify the dynamic, temporal aspects of the potential endocrine response to transportation. Furthermore, to discern the true response of animals to transportation they should be rested on the trailer before initiation of transportation to allow for recovery from the initial stimuli associated with the trailer loading process.

Concentrations of cortisol and epinephrine were greater in Temperamental bulls relative to Calm bulls prior to and following transportation; however, there were no differences between pre- and post-transportation concentrations of cortisol or epinephrine within groups. Differences in cortisol due to temperament are in concert with studies in mice in which aggressive mice strains have greater basal concentrations of cortisol than less aggressive strains (Shim et al., 2008). In regards to transportation, serum cortisol concentrations in mice which were transported for 3-4 hr were greater at the end of transportation when compared to non-transported mice (Shim et al., 2008). In

addition, there are variable findings as to cortisol concentrations being different in cattle before and after transportation (Blecha et al., 1984; Murata et al., 1985, 1987; Kenny and Tarrant, 1987; Tarrant et al., 1992; Buckham Sporer et al., 2008). Specifically, Blecha et al. (1984) did not detect a change in cortisol concentrations in steers transported for a 10-hr period compared to non-transported controls. They suggested that this was because cortisol concentrations increased during transportation when blood samples were not obtained, and by the time the transport concluded, cortisol concentrations had returned to pre-transportation values. Kenny and Tarrant (1987) found cortisol concentrations increased following a 1-hr transport, which further advocates that the response to transportation is more acute, with cattle acclimating to the procedure. However, transportation of cattle for 24 hr also increased cortisol concentrations in Friesian steers, an affect that was influenced by stocking density (Tarrant et al., 1992). In the current study the stocking density in the trailer was 235 kg/m^2 , which is less than half of the maximum (550 kg/m^2) suggested by Tarrant et al. (1992). Therefore, cortisol concentrations should not have been affected by stocking density. However, breed differences may affect basal concentrations of cortisol as the values observed in this study of Brahman bulls were lower than that for Aberdeen Angus and Friesian bulls as reported by Buckham Sporer et al. (2008).

Elevation of cortisol is often designated as an indicator of stress. However, epinephrine is produced in a parallel, often less-recognized response. Studies indicate that the production of cortisol via the hypothalamic-pituitary-adrenal axis is associated with perceived environmental stress (i.e. noise), while the sympathetic nervous system is

responsible for the response to transportation stress (neurogenic stress; Griffin, 1989; Mitchell et al., 1989; Minton, 1994). This suggests that both cortisol and epinephrine concentrations should be elevated in response to transportation stress. Interestingly, there is limited documentation of epinephrine concentrations in response to transportation. Odore et al. (2004) reported greater concentrations of epinephrine following transportation of 6-month old Blonde D'Aquitaine calves for 14 hr, with concentrations similar to those produced by Calm and Intermediate bulls in the current study.

The correlation of maximum and minimum rectal temperature was expected. Interestingly, there was a positive correlation between EV and maximum rectal temperature. This, along with the greater peak rectal temperature reached in Temperamental bulls, suggests that Temperamental bulls may exhibit greater temperature response to stressors compared with Calm and Intermediate bulls. The tendency for a correlation between pre-transport epinephrine and maximum rectal temperature is not surprising, as catecholamine concentrations have been implicated in stress-induced hyperthermia (Olivier et al., 2005). Similar to basal concentrations, pre- and post-transport concentrations of epinephrine and pre-transport concentrations of cortisol were correlated with EV.

CONCLUSION

In conclusion, temperament was a clear indicator of the body temperature response of bulls to transportation. Temperamental bulls had greater concentrations of

cortisol and epinephrine and also had elevated rectal temperature compared to the Calm bulls. These data elucidate dynamic changes in rectal temperature to various stimuli. Yet understandably, these data do not completely explain the relationship between stress hormone concentrations and rectal temperature, due to the timing of sampling for cortisol and epinephrine. Future studies could solve this problem by collecting samples for stress hormone analysis and rectal temperature at similar time points prior to, during, and after transportation.

CHAPTER IV
TEMPERAMENT INFLUENCES ENDOTOXIN-INDUCED CHANGES IN RECTAL
TEMPERATURE, SICKNESS BEHAVIOR, AND PLASMA EPINEPHRINE
CONCENTRATIONS IN BULLS

INTRODUCTION

Reducing the effects of disease is a major goal in the cattle industry as morbidity is more costly to producers than mortality due to: a) the expense of treating disease and b) the negative impact of disease on performance (Smith, 1998). Stress is a significant factor influencing immune function. Acute stress is not necessarily detrimental to the health of an animal, and may even elicit beneficial immune responses (Galyean et al., 1999; Dhabhar, 2002; Duff and Galyean, 2007; Sorrells and Sapolsky, 2007). However, chronic stress can negatively impact growth, reproductive, and immune functions (Moberg, 1987; Dobson et al., 2001; Shi et al., 2003; Silberman et al., 2003; Compas et al., 2004; Zhao et al., 2008). Therefore, the intricate relationship between stress hormones and the immune system is dependent upon the duration of stress and the influence of stress hormones on activation of the immune system.

The innate immune system can be activated by molecules or chemicals derived from pathogens that cause acute inflammation and infection (Andreasan et al., 2008; Männel, 2007). Application of an endotoxin (lipopolysaccharide, LPS; a component of the cell wall of gram negative bacteria) initiates an inflammatory response and increases stress hormone concentrations in mammals (Webel et al., 1997; Iwasaki et al., 2008;

Borghetti et al., 2009; Williams et al., 2009). For example, Williams et al. (2009) demonstrated that cortisol, epinephrine, and norepinephrine increased in young pigs in response to an i.v. LPS challenge.

Often unappreciated is the influence of temperament and stress responsiveness on livestock health and productivity. Temperament is defined as the fear response of cattle to humans or novelty (Fordyce et al., 1988). More stress responsive, temperamental cattle characteristically have greater basal concentrations of the stress hormones cortisol and epinephrine, depressed immune functions, a slower growth rate, and reduced carcass value (Curley et al., 2006,2008; King et al., 2006; Petherick et al., 2009). This study was designed to determine whether the temperament of young, growing Brahman bulls is related to the febrile response and the production of cortisol and epinephrine induced by an acute endotoxin challenge.

MATERIALS AND METHODS

Experimental Design

All experimental procedures were in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committees of Texas A&M University and the USDA. Bull calves (10 mo of age) from the Texas AgriLife Research Center's Brahman herd in Overton, TX were selected for use in this study based on their temperament score measured 28 d prior to weaning (133 ± 3 d of age). Temperament score (King et al., 2006) was an average of exit velocity (EV) and pen score (PS). Exit velocity is an

objective measurement that represents the rate of speed (m/s) of a calf traversing a distance of 1.83 m after its exit from a working chute (Burrow et al., 1988; Curley et al., 2006). Pen score (Hammond et al., 1996) is a subjective measurement obtained by separating cattle into small groups of three to five animals and scoring their reactivity to a human observer on a scale of 1 (calm, docile, approachable) to 5 (aggressive, volatile, crazy). Based on temperament score the 8 most Calm, 8 Intermediate, and 8 most Temperamental were selected from a pool of 60 bulls. However, during the experiment 1 Calm bull died (1 hour post-LPS), researchers intervened to prevent death of another Calm bull (3 hr post-LPS), and 1 Temperamental bull's catheter became dislodged (0 hr post-LPS). Therefore, only the data from 6 Calm (1.01 ± 0.16 EV and 1.00 ± 0.00 PS), 7 Temperamental (3.51 ± 0.25 EV and 5 ± 0.00 PS), and 8 Intermediate (1.59 ± 0.12 EV and 2.25 ± 0.16 PS) bulls are presented. Two days prior to initiation of the endotoxin challenge portion of the study bulls were fitted with rectal temperature recording devices (A HOBO Pro v2 Temp data logger probe; Part # U23-004, Onset Corp., Pocasset, MA) that measured rectal temperature continuously at 1-min intervals in the absence of a human operator. During these procedures cattle were restrained in a working chute for approximately 5 min. The factory calibrated rectal temperature recording devices were tested for accuracy upon receipt from the manufacturer. Rectal temperature recorders became displaced from some animals during the study; temperature data presented includes only those bulls that yielded a complete data set ($n = 5, 6,$ and 7 for Calm, Intermediate and Temperamental, respectively). On the day prior to the study bulls were fitted with jugular catheters. During these procedures cattle were restrained in a working

chute for approximately 5 to 10 min. Following these procedures bulls were moved to the facility that contained individual stalls (7 ft long x 2.5 ft wide) that housed the bulls through the duration of the study. Bulls were randomly placed in their stalls. During the challenge the bulls had *ad libitum* access to feed and water. The extension tubing of the catheter was extended above the stall to allow researchers to collect blood throughout the study without disturbing the calf, whether the calf was standing or lying down. Blood samples were collected and transferred into vacutainers containing no additive (serum) or tubes containing EDTA (plasma) every 30 min beginning 2 hr prior to and continuing 8 hr after administration of lipopolysaccharide (0.5 µg/kg BW LPS; *Escherichia coli* O111:B4; Sigma-Aldrich, St Louis, MO, USA). Blood samples transferred into vacutainers containing EDTA were processed immediately by centrifugation at 1500 x g for 10 min at 4°C. Blood samples transferred into vacutainers containing no additive remained on ice for 1 hr to allow samples to coagulate before processing by centrifugation at 1500 x g for 15 min at 4°C. Isolated plasma and serum samples were stored at -80°C until analysis for epinephrine and cortisol concentrations, respectively.

Sickness Behavior

Sickness behavior scores were assigned to animals at 30-min intervals from 0 to 6 hr post-LPS challenge, and were assigned by a single individual throughout the study. Bulls were scored on a scale of 1 (active or agitated) to 5 (lying on side with labored breathing; Table 1) based on the level of activity within their stall.

Assays for Cortisol and Epinephrine

Serum concentrations of cortisol were determined using a single antibody radioimmunoassay (DSL-2100; Diagnostic Systems Labs, Webster, TX) utilizing rabbit anti-cortisol antiserum coated tubes according to the manufacturer's directions (Burdick et al., 2009). The minimum detectable cortisol concentration was 1.2 ng/mL and the intra- and inter-assay coefficients of variance were 4.3% and 2.4%, respectively. Serum concentrations of cortisol were determined by comparison to a standard curve generated with known concentrations of cortisol and presented as the concentration in ng/mL.

Plasma concentrations of epinephrine were determined by enzyme immunoassay according to the manufacturer's directions (17-BCTHU-E02; Alpco Diagnostics, Boston, MA) by comparison of unknowns to standard curves generated with known concentrations of epinephrine (Burdick et al., 2009). Data are presented as pg/mL. The minimum detectable epinephrine concentration was 11 pg/mL and the intra- and inter-assay coefficients of variation were 3.7% and 7.4%, respectively.

Statistical Analysis

Prior to analysis, rectal temperature data were averaged into 30-min intervals. Rectal temperature, sickness behavior, cortisol, and epinephrine data were analyzed using the MIXED procedure of SAS (SAS, Inc., Cary, N.C.) specific for repeated measures with temperament, time, and time*temperament interaction included as fixed effects. Specific pre-planned comparisons were made using Fisher's Protected LSD with $P < 0.05$ considered significant.

Table 1. Sickness behavior score descriptions based on activity of Brahman bulls in response to administration of lipopolysaccharide.

Score	Behavior Description
1	Active or agitated
2	Appeared normal
3	Immobile with head distended
4	Clinical signs of sickness, increased respiration
5	Lying on side with labored breathing

RESULTS

Rectal Temperature

Prior to the administration of LPS, rectal temperature was greater in Temperamental bulls than Calm and Intermediate bulls ($P < 0.001$), with Intermediate bulls having greater rectal temperature than Calm bulls ($P = 0.05$). Rectal temperatures prior to the administration of LPS were not affected by time ($P > 0.05$; Figure 7). Rectal temperature increased in all bulls following administration of LPS, peaking around 210 min, with Temperamental bulls having the smallest increase in rectal temperature (relative to baseline values) compared to Calm and Intermediate bulls ($P < 0.001$).

Twenty-four hr (1440 min) after the administration of LPS, rectal temperature of Calm and Intermediate bulls had returned to baseline values; however, Temperamental bulls had lower rectal temperatures than Calm and Intermediate bulls ($P < 0.05$).

Sickness Behavior

Prior to the administration of LPS, sickness behavior scores were similar amongst temperament groups ($P > 0.05$; Figure 8). Following administration of LPS at time 0, sickness behavior scores increased in all bulls ($P < 0.001$). Calm calves had greater sickness behavior scores when compared to Intermediate ($P = 0.005$) and Temperamental bulls ($P < 0.001$). Additionally, Intermediate bulls had greater sickness behavior scores than Temperamental bulls ($P < 0.001$). There was a tendency for a time*temperament interaction ($P = 0.06$). Specifically, peak sickness behavior scores occurred at 0.5 hr post-LPS administration for Calm bulls, and at 1 hr post-LPS administration in Intermediate and Temperamental bulls. Peak sickness behavior scores were greater in Calm and Intermediate bulls compared to Temperamental bulls ($P < 0.001$ and $P = 0.007$ for Calm and Intermediate bulls, respectively).

Cortisol and Epinephrine

Prior to the administration of LPS, Temperamental bulls had greater concentrations of cortisol than Calm and Intermediate bulls ($P < 0.001$) as depicted by Figure 9. Cortisol concentrations tended to decrease from -2 hr to time 0 ($P = 0.07$).

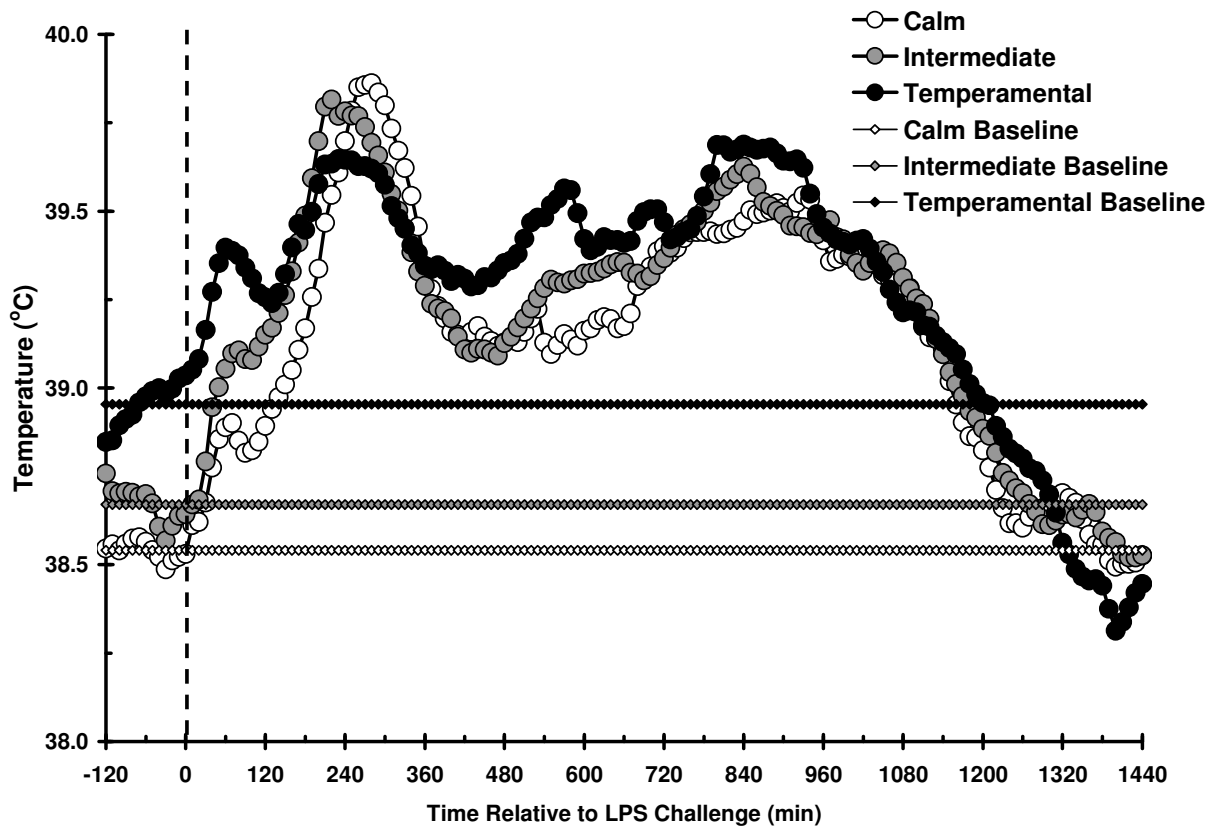


Figure 7. Rectal temperature response of Brahman bulls to an endotoxin (lipopolysaccharide, LPS; 0.5 ug/kg BW) challenge (Calm, $n = 5$, Intermediate, $n = 6$, and Temperamental, $n = 7$). Baseline lines represent pre-LPS averages ($SEM \pm 0.15$, 0.17, and 0.12 for Calm, Intermediate, and Temperamental, respectively). Rectal temperature increase in all bulls, with Temperamental bulls displaying a lower peak rectal temperature than Calm and Intermediate bulls ($P < 0.05$).

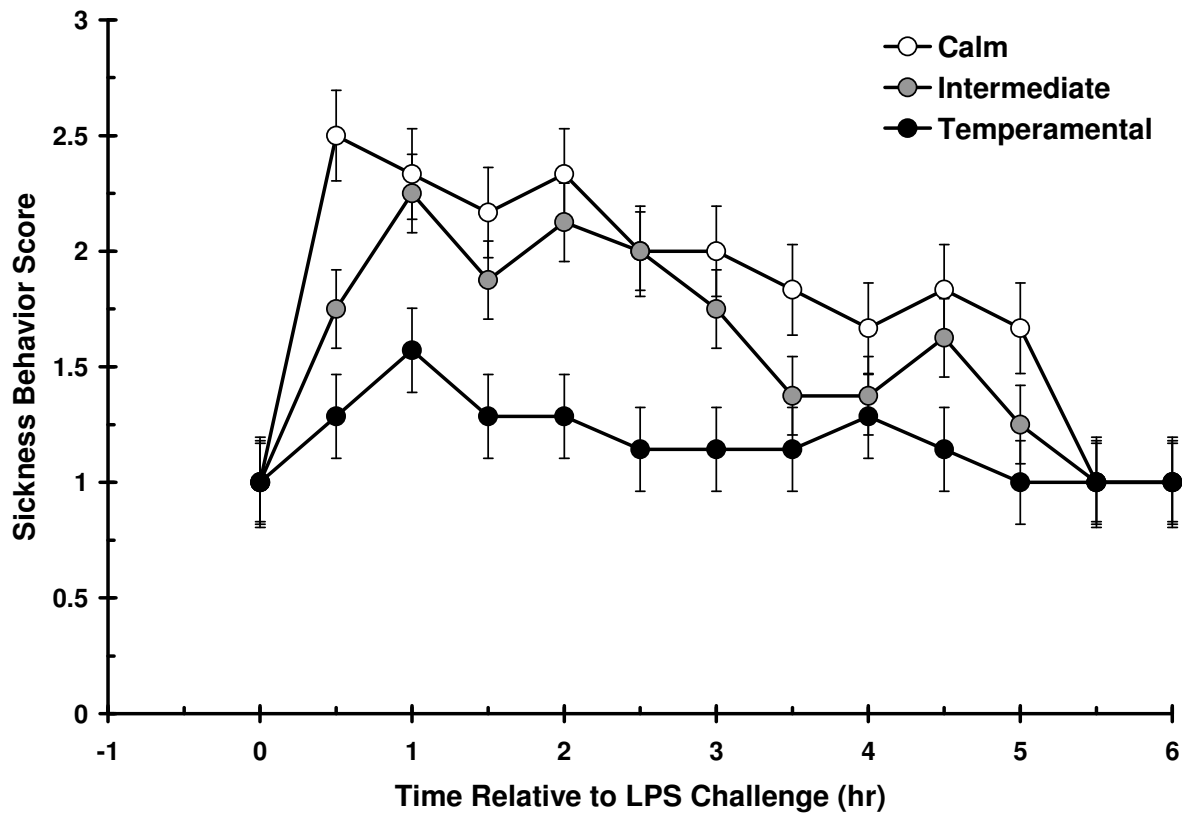


Figure 8. Sickness behavior response of Brahman bulls to an endotoxin (lipopolysaccharide, LPS; 0.5 ug/kg BW) challenge (Calm, $n = 6$; Intermediate, $n = 8$; and Temperamental, $n = 7$ bulls). Peak sickness behavior in Calm bulls occurred 0.5 hrs after LPS administration and was greater than the peak displayed by Intermediate and Temperamental bulls 1 hr post-challenge ($P < 0.05$).

Following administration of LPS at time 0, cortisol concentrations increased ($P < 0.001$) through 2 hr before declining, and were similar among temperament groups ($P = 0.80$). Prior to the administration of LPS, Temperamental bulls had greater concentrations of epinephrine than Calm and Intermediate bulls ($P < 0.001$) as depicted by Figure 10. There was a significant time effect ($P < 0.001$) and time*temperament interaction ($P = 0.014$) prior to the administration of LPS with epinephrine concentrations increasing from -2 to -1 hr in Calm ($P = 0.05$) and Temperamental ($P < 0.001$) bulls, and increasing from -2 to -0.5 hr in Intermediate bulls ($P = 0.02$). Relative to time 0, epinephrine concentrations did not change in response to the LPS challenge in Intermediate bulls ($P > 0.05$). Epinephrine concentrations tended to peak 1 hr after the administration of LPS in Calm bulls ($P = 0.06$). In contrast, epinephrine concentrations in Temperamental bulls decreased from time 0 to 0.5 hr after the administration of LPS ($P = 0.01$). Epinephrine concentrations then increased from 0.5 hr post-LPS, with higher concentrations of epinephrine at 1, 2, 3, 3.5, 4, 4.5, and 5.5 hr post-LPS compared to values at 0.5 hr ($P < 0.05$). Post-LPS administration, Temperamental bulls maintained greater epinephrine concentrations than either the Calm or the Intermediate bulls ($P < 0.05$).

DISCUSSION

These data demonstrate that temperament is related to the endocrine, behavioral, and physiologic responses of pre-pubertal Brahman bulls to endotoxin challenge. Rectal temperature, sickness behavior, cortisol, and epinephrine increased in response to

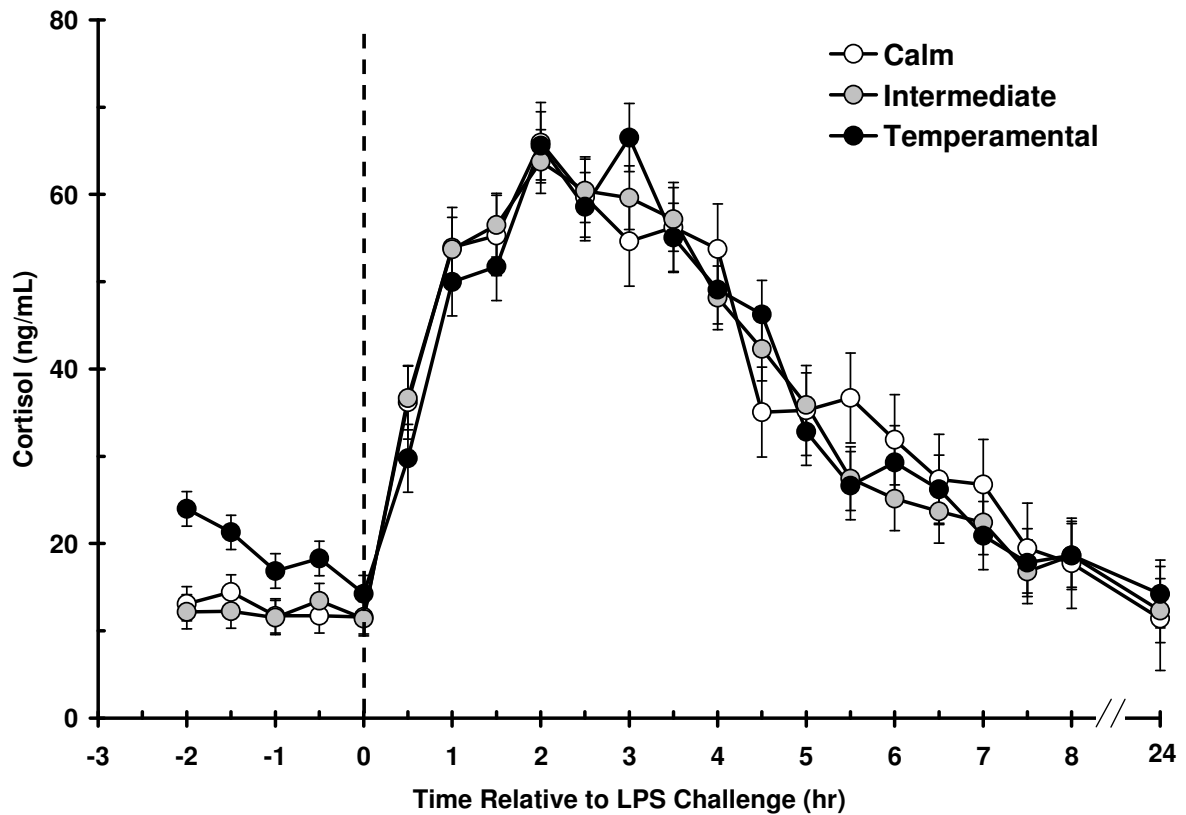


Figure 9. Serum cortisol response of Brahman bulls to an endotoxin (lipopolysaccharide, LPS; 0.5 ug/kg BW) challenge (Calm, n = 6; Intermediate, n = 8; and Temperamental, n = 7 bulls). Pre-LPS cortisol concentrations were greater in Temperamental bulls than Calm and Intermediate bulls ($P < 0.001$). Cortisol concentrations increased in all bulls in response to LPS ($P < 0.05$) but were not affected by temperament after the LPS challenge ($P > 0.05$).

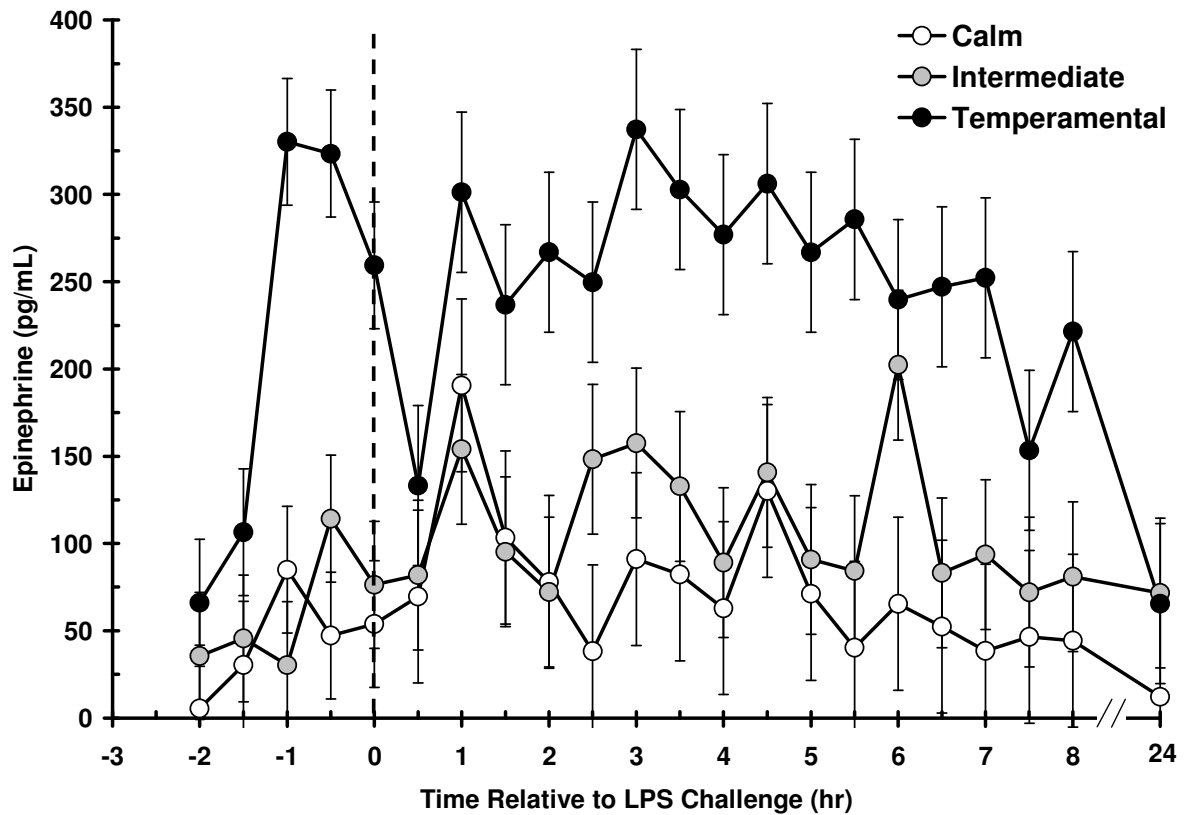


Figure 10. Serum epinephrine response of Brahman bulls to an endotoxin (lipopolysaccharide, LPS; 0.5 ug/kg BW) challenge (Calm, n = 6; Intermediate, n = 8; and Temperamental, n = 7 bulls). Pre-LPS, Temperamental bulls had greater concentrations of epinephrine compared to Calm and Intermediate bulls ($P < 0.001$). Epinephrine concentrations tended to increase in response to LPS in Calm bulls ($P = 0.06$), but did not change in Intermediate bulls ($P > 0.05$). In Temperamental bulls epinephrine concentrations decreased from time 0 to 0.5 hrs after challenge, but increased from 0.5 hrs through 7 hr post-challenge ($P < 0.05$). Post-LPS, Temperamental bulls maintained greater concentrations of cortisol than either the Calm or the Intermediate bulls ($P < 0.05$).

administration of LPS. Specifically, temperament differentially affected the rectal temperature, sickness behavior, and epinephrine, but not cortisol, responses to LPS challenge.

No differences in baseline body temperature were found in rats selectively bred to differ in behavior (high-anxiety and low-anxiety behavior lines; Liebsch et al., 1998). However, documentation regarding the potential for temperament to influence rectal temperature is limited in cattle. In the present study there was an effect of temperament on pre-LPS rectal temperatures in bulls, with Temperamental bulls having greater rectal temperatures than Intermediate and Calm bulls. This is similar to our prior study which demonstrated that rectal temperature was greater in Temperamental bulls than Calm and Intermediate bulls immediately after loading into a trailer (Burdick et al., 2010).

An increase in body temperature has been utilized as a characteristic response of cattle to LPS challenge, and as a sign of inflammation (Elsasser et al., 1996; Bieniek et al., 1998; Jacobsen et al., 2005; Borderas et al., 2008; Reuter et al., 2008; Carroll et al., 2009; Waggoner et al., 2009a,b). An increase in body temperature in response to LPS is stimulated by pro-inflammatory cytokines, mainly tumor necrosis factor alpha (TNF- α), interleukin 1 beta (IL-1 β) and IL-6 (Dinarelli et al., 1996; Steiger et al., 1999; Black, 2002). In response to LPS peak rectal temperatures in the Brahman bulls used in this study were attained within 3.5 hr which was earlier than the 6 hr required for Holstein cows to attain peak rectal temperatures post-LPS challenge (Jacobsen et al., 2005). Peak rectal temperatures were also reached 1 hr earlier in the Brahman bulls than in a study where Angus steers did not reach peak rectal temperatures until 4.5 hr post-LPS (Carroll

et al., 2009). However, the Jacobsen study (2005) used a lower dose of LPS (0.1 $\mu\text{g/kg}$) and was performed in mature dairy cows, in contrast to the current study which utilized a higher LPS dose (0.5 $\mu\text{g/kg}$) in young, pre-pubertal Brahman beef bulls. The study on Angus steers (Carroll et al., 2009) used a larger dose of LPS (2.5 $\mu\text{g/kg}$) than the current study. Therefore, although similar febrile responses have been demonstrated in cattle studies, the dose administered or the type of animal model utilized can influence the time to reach peak values and the duration in which rectal temperatures are elevated.

Twenty-four hours after the administration of LPS, the rectal temperature of Calm and Intermediate bulls had returned to baseline. However, Temperamental bulls had significantly lower rectal temperature compared to baseline values. The mechanism for LPS-induced hypothermia in cattle is unclear; however, it may be associated with dehydration. Several rodent models have been employed to study LPS-induced hypothermia (Ochalski et al., 1993; Roth et al., 2009). However, in many rodent models hypothermia can precede fever, or is the only temperature response. The response in rodents is reported to be dependent on the dose of LPS administered (Ochalski et al., 1993; Roth et al., 2009). This is not what was seen in the current study, in which hyperthermia was followed by a period of LPS-induced hypothermia. Most reports using a bovine model only present data through 8-12 hr post-LPS administration, and therefore investigators did not report any subsequent changes in body temperature (Reuter et al., 2008; Waggoner et al., 2009b; Carroll et al., 2007).

Application of the rodent models enabled detection of interactions between cytokines, prostaglandins, and lipid mediators, each of which have a role in the

regulation of hypothermia (Ochalski et al., 1993; Roth et al., 2009; Steiner et al., 2009). It has been suggested that the capability to minimize or lessen the increase in body temperature in response to an LPS increases survival (Romanovsky et al., 1998). This may be a factor in this study, as all of the Temperamental and Intermediate bulls survived, yet one of the Calm bulls died, and the authors intervened in order to prevent the death of another Calm bull. Future studies are needed to determine the mechanisms resulting in LPS-induced changes in body temperature, particularly in cattle. The elevated stress hormones in more Temperamental cattle may serve as a protective mechanism when challenges with endotoxin occur.

Changes in body temperature in response to LPS may involve catecholamines within the central nervous system (Jüttler et al., 2007; Tolchard et al., 2009). Tolchard et al. (2009) demonstrated that agonists for the α_2 -adrenergic receptor, the receptor that inhibits responses to the catecholamines, epinephrine, and norepinephrine, inhibited LPS-induced hypothermia, suggesting a role for catecholamines. This is interesting as epinephrine concentrations were greater in Temperamental bulls prior to and following the administration of LPS, as discussed in more detail below. Change in body temperature induced by substances such as LPS is regulated by thermosensitive central nervous system neurons that innervate the hypothalamus (Jüttler et al., 2007). In mice, Jüttler et al. (2007) demonstrated that the neurons responsible for mediating actions of pro-inflammatory cytokines within this region contain the active transcription factor nuclear factor- κ B (NF- κ B), which is responsible for downstream actions of LPS, including an increase in inflammatory response. It will be necessary to determine if the

cytokine response to LPS is affected by temperament, thereby explaining the differences in rectal temperature observed in Temperamental bulls (Kovalovsky et al., 2000).

Animals respond behaviorally to illness in various ways, simultaneously with physiological changes, in order to help the animal cope with the illness (Borderas et al., 2008). These behaviors include weakness, malaise, depression, lethargy, and decreases in eating and drinking (Dantzer, 2001, 2004). Sickness behavior is induced by pro-inflammatory cytokines including IL-1 β , IL-6, TNF- α and interferon- γ (IFN- γ ; Dantzer et al., 2001). The systemic administration of these cytokines results in the onset of sickness behavior in both humans and in animals, with these responses mimicked by the administration of LPS (Dantzer et al., 2001). Whereas there are several studies utilizing rodent and human models, there are limited studies describing the effect of LPS on sickness behavior in cattle.

A study in young dairy calves found an increase in the amount of time calves were lying inactive, and found a decrease in the time eating hay and ruminating 2 hr before and after the peak in rectal temperature in response to low doses of LPS (0.025 and 0.05 $\mu\text{g/kg BW}$; Borderas et al., 2008). In the current study, calves of Calm and Intermediate temperament spent more time lying, as indicated by their sickness behavior scores. An animal lying on their side may be a mechanism for which an animal increases heat loss through transfer of heat from the body to the ground. Our observations that Temperamental bulls had a lower sickness behavior score than did either Intermediate or Calm bulls suggest that temperament can influence sickness behavior in cattle. Several research articles have put forth the concept that sickness

behavior is not a manifestation of innate behavioral or psychological weakness but is an expression of a motivational state (Dantzer, 2001, 2004; Dantzer and Kelly, 2007).

Specifically, Dantzer (2001) stated that motivation to exhibit fear behavior competes with sickness behavior, with fear taking priority over sickness-related behavior.

Therefore, it may be that Temperamental bulls did not appear as sick as Intermediate and Calm bulls due to an increase in fear behavior to their environment and the human workers. Further research is needed in order to elucidate the mechanisms by which cytokines (both peripheral and in the brain) regulate sickness behavior, and the mechanisms by which temperament influences this response.

Peripheral blood concentrations of cortisol and epinephrine prior to the administration of LPS were greater in Temperamental bulls than in Intermediate and Calm bulls. This is in agreement with our previous studies (Curley et al., 2006, 2008; King et al., 2006) and studies conducted by others (Fell et al., 1999), in which basal concentrations of cortisol and epinephrine were greater in Temperamental bulls. It is unclear as to the exact causes for the difference in cortisol concentrations in Calm versus Temperamental cattle. Grandin (1997) suggests that temperament is a genetic factor due to its heritability. Burrow (2001) found flight speed (exit velocity), a measure of temperament, to be moderately heritable (0.40-0.44) in Australian Belmont Red cattle. Quantitative trait loci that may influence temperament have also been discovered (Gutiérrez-Gil et al., 2008). However, temperament can be reduced with repeated handling (Curley et al., 2006; Fell et al., 1999; Burrow and Dillon, 1997) which suggests a complex interaction between genetics and environment resulting in changes in

physiology. Additionally, cortisol concentrations are known to be heritable (Levene and Workman, 1972; Meikle et al., 1988). In humans, morning plasma cortisol concentrations had a heritability of 0.45, with unbound (free or active) cortisol having a higher heritability of 0.51 (Meikle et al., 1988), which suggests a genetic basis for cortisol concentrations. Studies in both humans and livestock have indicated a relationship between cortisol output, including individual variability in cortisol secretion, with disease risk (Federenko et al., 2004; Mormède, 2007). Although the cortisol response to LPS challenge was not affected by temperament, the difference in basal cortisol concentrations due to temperament prior to the administration of LPS may have influenced the overall response to LPS challenge.

Cortisol is a potent anti-inflammatory hormone that is secreted in response to endotoxin challenge. Previous studies in cattle have established that LPS induces an increase in cortisol concentrations (Carroll et al., 2009; Waggoner et al., 2009a,b; Kahl et al., 2009). The cortisol response to LPS in this study was similar to that described by Carroll et al. (2009). In contrast, Kahl et al. (2009) utilizing pubertal beef heifers, found that LPS stimulated an increase in cortisol concentrations 2 hr after administration, and concentrations remained elevated through 7 hr post-challenge. It should be noted that Kahl et al. (2009) used a 5-fold larger dose (2.5 µg/kg) of LPS than was used in the current study.

Bos indicus cattle, such as those used in this study, appear to be more sensitive than *Bos taurus* cattle to endotoxin. Other studies suggested a breed difference in response to immunological stimuli (Carroll et al., 2007; Blecha et al., 1984). For

example, Blecha et al. (1984) reported a greater response to phytohemagglutinin skin test by Angus steers than by Brahman x Angus cross steers. Compared to the current study, Carroll et al. (2009) delivered a 5-times greater dose of LPS to purebred Angus steers (of undefined temperament), yet produced a cortisol response similar in duration and magnitude to the response which we observed for purebred Brahman bulls. This suggests that the *Bos taurus* genotype may convey added protection or resistance to the detrimental actions of LPS. Therefore, breed of cattle, as well as temperament of the cattle should be considered during the design and the comparison of results of experiments in which LPS is utilized to stimulate the innate immune system as well as an inflammatory response.

While there was no difference among the temperament groups with respect to the cortisol response to endotoxin challenge, there were clear differences due to temperament in the epinephrine response to LPS challenge. Specifically, epinephrine concentrations in Temperamental bulls did not increase in response to LPS compared to time 0 (prior to the administration of LPS). However, epinephrine concentrations decreased from time 0 to 0.5 hr post-challenge before increasing at 1 hr post-challenge. Therefore, in response to an endotoxin challenge, temperament may differentially affect the response of the adrenal medulla, but not the adrenal cortex. This is supported by the report that more aggressive mice have greater concentrations of phenylethanolamine N-methyltransferase (PNMT), the enzyme that converts norepinephrine to epinephrine, in the adrenal medulla, with no differences in cortisol production (Sorensen et al., 2005). However, there is no prior documentation on the effect of temperament on the stress

hormone response to LPS challenge. Based on our survey of the relevant literature this appears to be the first or at least among the earliest demonstration of the temporal epinephrine response of Brahman cattle to an endotoxin challenge. The peak epinephrine response of the Calm bulls is similar in magnitude to the response in young pigs, but occurred 45 min later (Williams et al., 2009). The epinephrine concentrations post-LPS challenge in Calm and Intermediate bulls were lower than that induced by venipuncture stimulation and transportation of Red Angus bulls (201.5 and 219.8 pg/mL; Rulofson et al., 1988). However, epinephrine concentrations in Temperamental bulls post-LPS challenge were greater than those found in the study by Rulofson et al. (1988).

Studies have suggested that epinephrine tolerance protects mice and dogs against endotoxin-induced shock (Motsay et al., 1971; Baykal et al., 1999). Taking into consideration that the Temperamental bulls had greater basal concentrations of cortisol when compared to bulls of Calm and Intermediate temperament, the greater basal concentrations of cortisol may protect Temperamental bulls. A recent study by Frank et al. (2009) found that pre-treatment with glucocorticoids prior to stimulation with LPS increased pro-inflammatory cytokines in the hippocampus, which may have priming actions of the nervous system and mediate the manner in which the mice respond to the inflammatory stimuli. However, this does not explain why epinephrine concentrations did not increase in response to the challenge with LPS. It is possible that epinephrine failed to increase due to negative feedback mechanisms imposed by higher basal concentrations of epinephrine in Temperamental bulls, yet further research is needed to identify the specific mechanisms. The endocrine response of Temperamental bulls to

LPS is unique and requires more attention to elucidate the mechanism in which temperament differentially affects the production of stress hormones by the adrenal gland.

In conclusion, LPS-induced the secretion of the stress hormones cortisol and epinephrine, increased rectal temperature, and sickness behavior scores. While rectal temperature, sickness behavior, and epinephrine differentially responded based on temperament, temperament did not affect the response of the adrenal cortex to endotoxin challenge. It is not yet clear whether this differential innate response to LPS (i.e., diminished febrile response, sickness behavior response, and failure to produce an epinephrine response) is beneficial or potentially detrimental to either the near or long-term health of Temperamental bulls. Future studies should determine if temperament affects properties of both the innate and adaptive immune response to LPS challenge. A clearer understanding of the inter-relationship between stress hormones and the inflammatory process may lead to methods of early intervention to minimize the debilitating impacts of illness on growth and productivity.

CHAPTER V

INFLUENCE OF TEMPERAMENT AND TRANSPORTATION ON PHYSIOLOGICAL AND ENDOCRINOLOGIC PARAMETERS IN BULLS

INTRODUCTION

Animal temperament, and its influence on health and performance, is a growing area of interest in the cattle industry. In cattle, temperament is defined as the reactivity, or fear response, to humans or novel environments (Fordyce et al., 1988). Cattle with poor temperaments have been demonstrated to exhibit poor growth (average daily gain) and carcass traits while having an impaired immune function (Voisinet et al., 1997; Fell et al., 1999; Mondal et al., 2006; Oliphint et al., 2006). Additionally, measures of temperament, including exit velocity (Burrow et al., 1988) and pen score (Hammond et al., 1996), have been correlated with concentrations of stress hormones. Specifically, more excitable or temperamental cattle have greater basal concentrations of cortisol and epinephrine (Schuehle et al., 2005; King et al., 2006; Curley et al. 2006a,b, 2008; Burdick et al., 2010). Due to having greater basal concentrations of stress hormones, it is possible that temperamental cattle exhibit different responses to stress.

Transportation is a common management procedure that young beef cattle typically experience. Divergent conclusions regarding the effect of transportation on secretion of the stress-related hormone cortisol have been reported (Murata et al., 1987; Blecha et al., 1984; Buckham Sporer et al., 2007; Burdick et al., 2010). For example, Murata et al. (1987) found an increase in cortisol following a 4-hr transport. However,

in studies utilizing longer periods of transportation (8 to 9 hr) cortisol concentrations did not differ between 1) pre- and post- transportation concentrations (Burdick et al., 2010; Brahman bulls) or 2) transported calves and non-transported control calves (Blecha et al., 1984; Angus and Angus-Brahman cross calves). Both studies suggested that cortisol concentrations increased in response to transport yet decreased by the time the post-transport samples were obtained.

Stress has been demonstrated to be immunosuppressive. For example, glucocorticoids are known to suppress many immune functions including but not limited to suppressing maturation, differentiation, and proliferation of all immune cells, inducing apoptosis of lymphocytes, reducing chemotaxis of leukocytes, and decreasing the number of circulating leukocytes (Martin, 2009). Specifically in response to transportation, Murata et al. (1985) found an increase in lymphocyte proliferation at 6 hr following a 1-hr transport, a condition which recovered 24 hr post-transportation. Similarly, neutrophil activity was enhanced 6 hr following transportation, but recovered by 24 hr post-transportation. In a later study, Murata et al. (1987) found leukocytosis, caused by neutrophilia, immediately following the end of a 4-hr transport of castrated Holstein calves. Transportation also decreased basal proliferation of lymphocytes following transportation. In response to a longer transportation (10 hr), Blecha et al. (1984) also found neutrophilia and an increase in leukocytes at unloading. However, there was no effect of transportation on the *in vivo* cell mediated response to phytohemagglutinin (PHA). Therefore, there are conflicting results as to the effect of transportation on lymphocytes, which may vary depending on the length of transport.

Temperament has also been noted to inhibit immune function (Oliphint et al., 2006); however, this documentation is limited.

This study was designed to collect samples during transport, in the absence of human presence, utilizing automatic sampling devices (IceSamplerTM). These sampling devices allowed for the determination in ‘real-time’ whether endocrine indices of stress responsiveness change during transportation, and if these changes were related to temperament. Additionally, samples were collected to determine the influence of temperament on proliferation, IgM production, and cytokine gene expression in isolated peripheral blood mononuclear cells (PBMCs) in response to transportation.

MATERIALS AND METHODS

Experimental Design

Bulls (10 mo of age) from the Texas AgriLife Research Center’s purebred Brahman herd in Overton, TX were selected for use in this study based on their temperament score measured 28 d prior to weaning (163 ± 2 d of age). Temperament score (Curley et al., 2006a; King et al., 2006) was an average of exit velocity (EV) and pen score (PS). Exit velocity is an objective measurement that records the rate (m/s) at which cattle exit a working chute (Burrow et al., 1988; Curley et al., 2006a). Pen score (Hammond et al., 1996) is a subjective measurement in which cattle are separated into small groups of three to five and their reactivity to a human observer scored on a scale of 1 (calm, docile, approachable) to 5 (aggressive, volatile, crazy). Based on temperament score the 7 most Calm (temperament score = 0.84 ± 0.03) and the 8 most Temperamental

(temperament score = 3.37 ± 0.18) were selected from a pool of 60 bulls. Prior to transportation bulls were fitted with heart rate monitors, jugular catheters, and rectal temperature recording devices (A HOBO Pro v2 Temp data logger probe; Part # U23-004, Onset Corp., Pocasset, MA; Burdick et al., 2010) that measured rectal temperature continuously at 1-min intervals in the absence of a human operator. The factory-calibrated rectal temperature recording devices were tested for accuracy upon receipt from the manufacturer. Data collection for rectal temperature and heart rate commenced following the loading of the bulls into a trailer with individual stalls. Following placement of measurement devices, bulls were loaded onto a trailer with individual stalls. Once in their stalls the catheter extensions were attached to an automated IceSamplerTM device (IceRobotics, Roslin, Midlothian, Scotland UK). Each device was pre-programmed to pull a waste collection, a sample collection, and flush the line with heparinized saline at 15- and 30-min intervals. The trailer remained stationary for 120 min to allow bulls to acclimate to their stall. The 120-min acclimation period also allowed for the subsequent discrimination of the response to transportation versus the combined response of loading plus transportation. After initiation of transportation at time 0, bulls were transported for 240 min (390 km roundtrip, 91 km/hr average speed). Whole blood was collected into heparinized syringes by the IceSamplerTM device, which was kept cold by surrounding the device with ice packs. Immediately after the end of transportation heparinized syringes were collected from the IceSamplerTM and centrifuged at $1,500 \times g$ at 4°C . Isolated plasma was stored at -80°C until analysis for cortisol, epinephrine, and norepinephrine. Plasma creatinine concentrations were also

determined to correct for possible sample dilution by the anticoagulant solution during collection of samples by the IceSampler™ device. Therefore, the data for each stress-related hormone (e.g., cortisol, epinephrine and norepinephrine) is presented as a ratio of the concentration of the hormone relative to the concentration of creatinine in each blood sample. Additionally, a separate whole blood sample was collected and transferred to a vacutainer containing EDTA prior to the attachment and following disconnection of the catheter extensions from IceSampler™ device for the isolation of PBMCs

Cortisol

Plasma concentrations of cortisol were determined using a single antibody radioimmunoassay (DSL-2100; Diagnostic Systems Labs, Webster, TX) utilizing rabbit anti-cortisol antiserum coated tubes according to the manufacturer's directions (Burdick et al., 2009). All samples were analyzed in one assay. The minimum detectable cortisol concentration was 1.2 ng/mL and the intra-assay coefficient of variation was 1.6%. Plasma concentrations of cortisol were determined by comparison to a standard curve generated with known concentrations of cortisol.

Epinephrine and Norepinephrine

Plasma concentrations of epinephrine and norepinephrine were determined by enzyme immunoassay according to the manufacturer's directions (17-BCTHU-E02; Alpco Diagnostics, Boston, MA; Burdick et al., 2009) by comparison of unknowns to standard curves generated with known concentrations of epinephrine and

norepinephrine. The minimum detectable epinephrine concentration was 11 pg/mL and the intra- and inter-assay coefficients of variation were 2.2% and 2.6%, respectively. The minimum detectable norepinephrine concentration was 44 pg/mL and the intra- and inter-assay coefficients of variation were 6.6% and 7.8% respectively.

Creatinine

Plasma concentrations of creatinine were determined by a quantitative colorimetric assay (DICT-500; BioAssay Systems, Hayward, CA) according to the manufacturer's directions. Plasma concentrations of creatinine were determined by comparison of unknowns to standard curves generated with known concentrations of creatinine.

PBMC Isolation

The PBMCs were isolated using density gradient centrifugation using Ficoll-Paque Plus (Cat. No. 17-1440-03; VWR, West Chester, PA, USA). Erythrocytes were lysed using a 0.2% NaCl solution followed by addition of 1.6% NaCl to achieve 0.9% salinity. Isolated PBMCs were resuspended in media containing DME/F12, 10% Horse Serum, 2 mM L-glutamine, 50 U/mL penicillin, 50 U/mL streptomycin, and 10 μ M 2-mercaptoethanol. Horse serum was used rather than fetal bovine serum in order to avoid possible cross-reactivity of bovine serum albumin in fetal bovine serum with the antibodies in the IgM ELISAs. Six million cells were used immediately for cell culture and the remaining cells were frozen at -80°C until RNA extraction.

PBMC Culture, Proliferation Assay and IgM ELISA

Isolated PBMCs were plated at a concentration of 1×10^5 cells per well in the presence of Concanavalin A (ConA; 0, 2.5, 5, and 10 $\mu\text{g/mL}$; Sigma Aldrich, St. Louis, MO) and incubated (37°C and 5% CO_2) for 96 hr. Following incubation, the extent of proliferation was determined using the Cell Titer 96™ proliferation assay according to the manufacturer's instructions (Promega, Madison, WI).

In duplicate cultures, plates were frozen at -20°C until determination of culture IgM production. Culture (cellular and supernatant) concentrations of IgM were determined using a double-antibody sandwich ELISA specific for bovine IgM (Bethyl Laboratories, Montgomery, TX). Culture concentrations were determined by comparison to a standard curve of known concentrations of bovine IgM and are expressed as the concentration in ng/mL .

PBMC RNA Extraction and Quantitative Real Time RT-PCR Analysis

Total RNA was extracted from PBMCs using Invitrogen's PureLink Micro-to-Midi RNA Purification System according to the manufacturer's instructions (Cat. No. 12183-018; Invitrogen, Carlsbad, CA, USA). Quality and quantity of RNA in elutes were determined using a spectrophotometer. Extracted RNA was treated with DNase I and cDNA synthesis was performed (all reagents from Invitrogen). Bovine-specific primers were designed from using Primer Express Software (Applied Biosystems, Foster City, CA, USA) and were ordered from Sigma Genosys (Table 2). Reactions containing

20 ng of cDNA were amplified in triplicate 25 µL reactions containing 2x SYBR Green PCR Mastermix (Applied Biosystems) and 6.25 nM of each primer. The 7900HT Fast Real-Time PCR System (Applied Biosystems) was used with the following conditions: heat inactivation at 50°C for 15 s, denaturation at 95°C for 10 min and annealing/extension at 56°C for 1 minute for 40 cycles, and dissociation curves obtained from 60°C to 95°C.

Statistical Analysis

All PCR reactions were normalized to bovine beta actin by dividing the Ct values for the gene of interest by the Ct value for beta actin. Relative differences were then calculated using the $2^{-\Delta\Delta C_T}$ method (Livack and Schmittgen, 2001) to determine fold change in gene expression compared to pre-transportation samples. There was no difference in expression of beta actin over time.

Prior to analysis, cortisol, epinephrine, and norepinephrine were averaged over 15- or 30-min intervals. Similarly, rectal temperature and heart rate were averaged over 15-min intervals. All data were analyzed using the MIXED procedure of SAS specific for repeated measures (SAS Inst., Inc., Cary, NC). Sources of variation included temperament, time and their interactions. Specific treatment comparisons were made using Fisher's Protected Least Significant Difference, with $P < 0.05$ considered significant.

Table 2. Primers used for quantitative real-time RT-PCR to determine gene expression of pro-inflammatory cytokines and associated receptors pre- and post-transportation^{1,2}.

	Forward Primer 5'→3'	Reverse Primer 5'→3'
TNF-α	CCGGTGGTGGGACTCGTAT	GCTGGTTGTCTTACAGCTTCACA
TNF-α R1	TACATCTCCTGTGACCGGTC	GCTGGCTTCCCACTTCTGAAC
TLR4	AATGGCAGGCAACTCTTTTCA	GGGCTACCTGTTCCAGTTGACA
IL-2	TCCAAGCAAAAACCTGAACC	CAGCGTTTACTGTTGCATCATC
IL-6	AAACCGAAGCTCTCATTAAGCG	TGGAAGCATCCGTCCTTTTC
IL-10	GCCTTGTCGGAAATGATCCA	TCAGGCCCGTGGTTCTCA
GR	TCAACTTGGCGGATCATGAC	CATTTCACGGCTGCAATCAC
Beta Actin	CGCCATGGATGATGATATTGC	AAGCCGGCCTTGACAT

¹Primers were designed using Primer Express Software (Applied Biosystems, Foster City, CA) and were ordered from Sigma Genosys.

²Abbreviations: Tumor necrosis factor- α (TNF- α); (Tumor Necrosis Factor- α receptor 1 TNF- α R1); Toll-like receptor 4 (TLR4); Interleukin-2 (IL-2); Interleukin-6 (IL-6); Interleukin-10 (IL-10); Glucocorticoid receptor (GR)

RESULTS

Rectal Temperature and Heart Rate

Rectal temperature increased (Figure 11; $P < 0.01$) in both Calm and Temperamental bulls throughout the experiment (prior to and during transportation). The increases in rectal temperature during the study may be partially explained by the temporal elevation in ambient temperature as these indices were highly correlated ($r = 0.73$ and $r = 0.72$ for Calm and Temperamental bulls, respectively; $P < 0.001$). Rectal temperature during the study was not affected by temperament ($P = 0.57$).

Heart rate remained steady in Calm bulls throughout the study and was not affected by time (Figure 12; $P > 0.05$). In contrast, the heart rate of Temperamental bulls fluctuated throughout the experiment ($P < 0.01$) resulting in a time by temperament interaction ($P = 0.04$).

Cortisol, Epinephrine, and Norepinephrine Concentrations

Cortisol concentrations increased in Calm bulls ($P < 0.05$) in response to the initiation of transportation, and remained elevated throughout transportation (Figure 13A). In contrast, cortisol concentrations in Temperamental bulls did not change in response to transportation ($P > 0.05$). Concentrations were not affected by temperament ($P = 0.26$) but there was a temperament by time interaction as described above ($P = 0.01$).

Epinephrine concentrations in Calm bulls remained relatively constant throughout the experiment, whereas the epinephrine concentrations in Temperamental bulls decreased ($P < 0.05$) throughout the experiment (Figure 13B). The concentrations of cortisol and

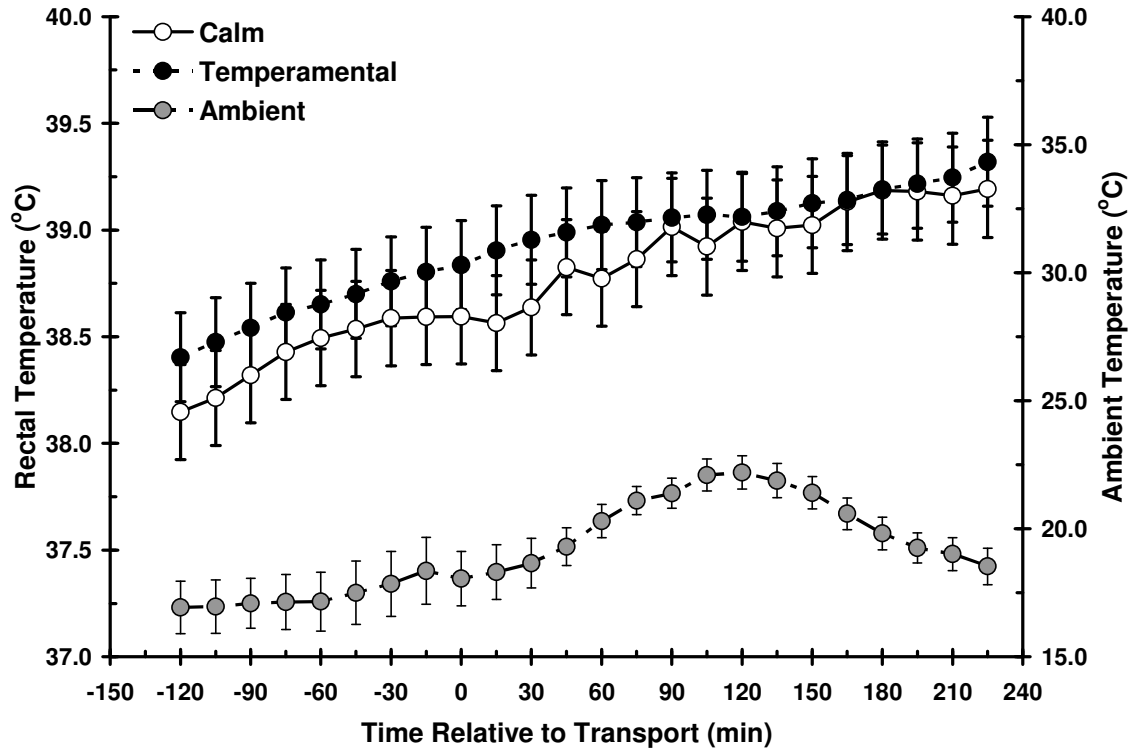


Figure 11. Rectal and ambient temperature prior to and during transportation of Brahman bulls for 4 hr. Data presented for Calm ($n = 7$) and Temperamental ($n = 8$) bulls. Rectal temperature increased throughout the study and was not affected by transportation ($P > 0.05$) or temperament ($P > 0.05$).

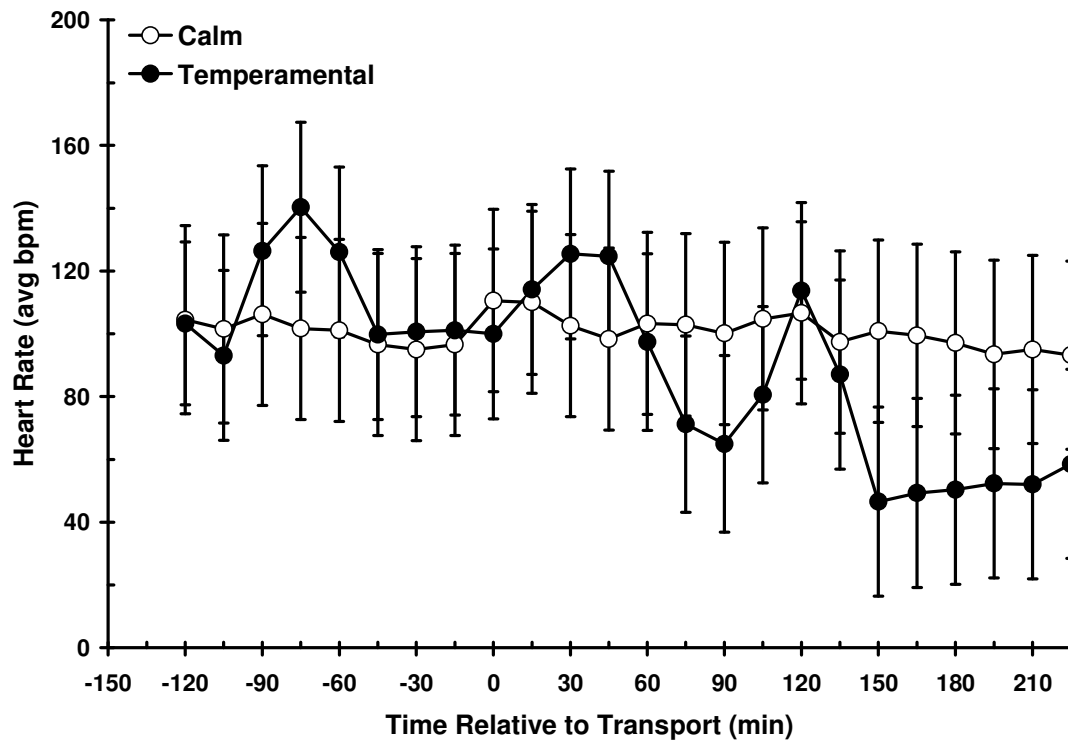
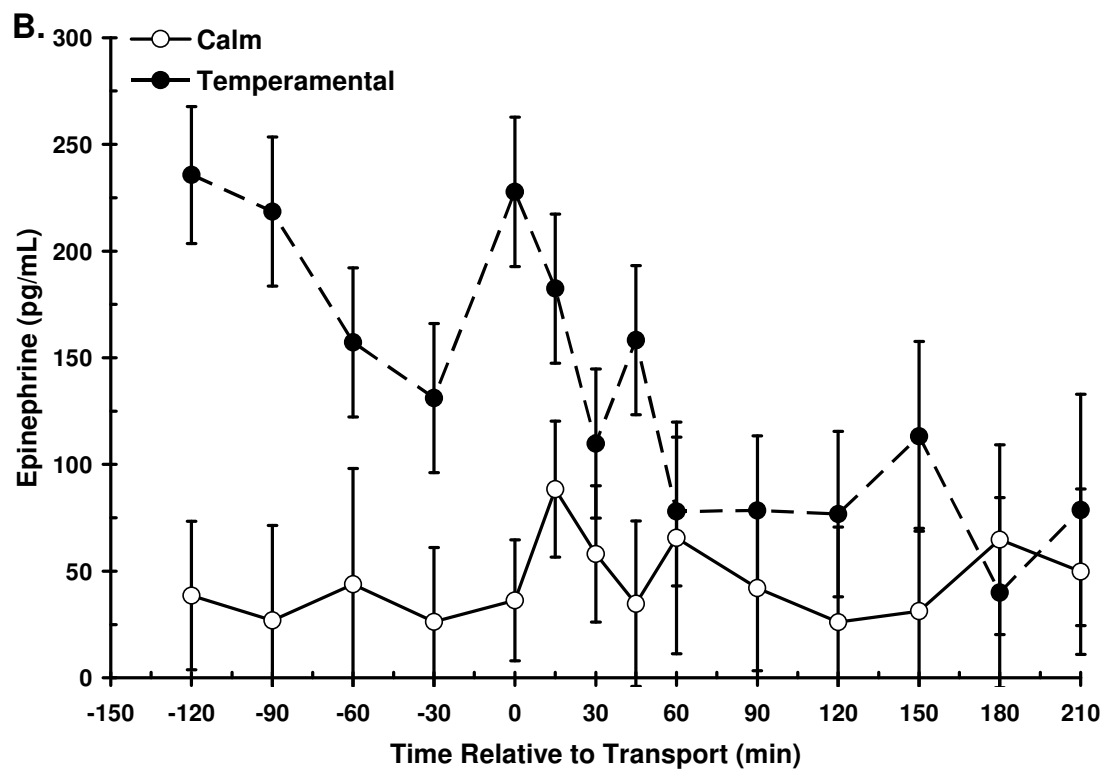
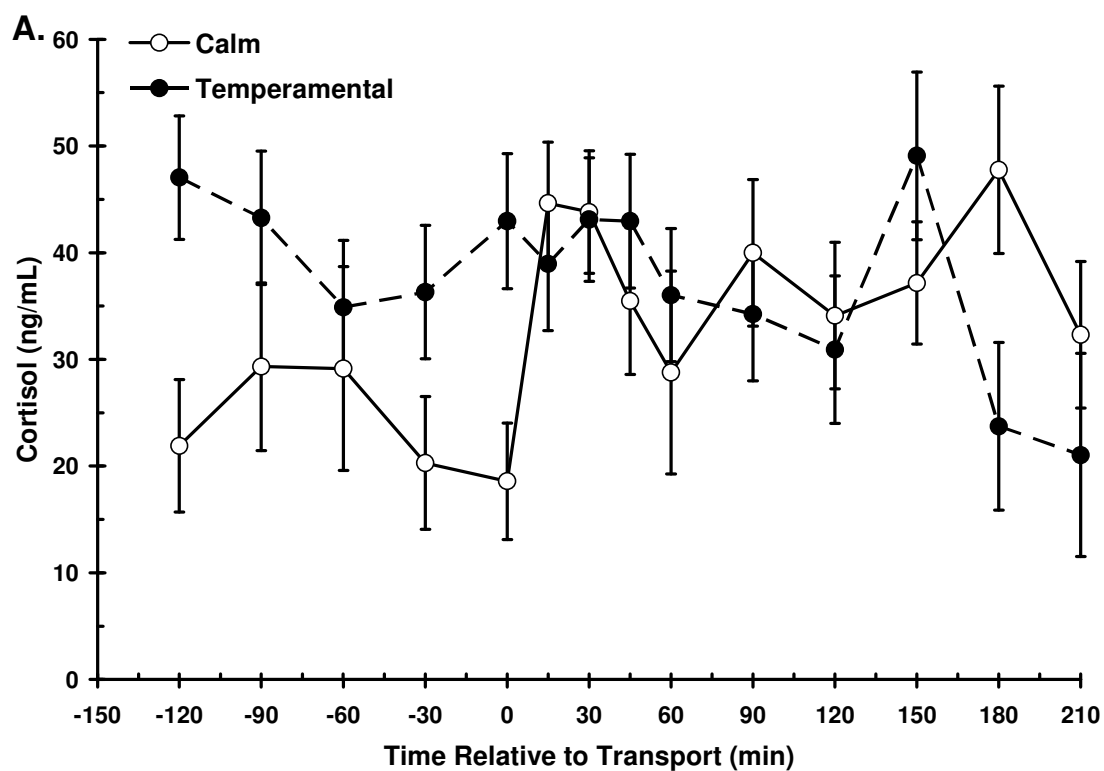


Figure 12. Heart rate prior to and during transportation of Brahman bulls for 4 hr. Data presented for 4 Calm and 4 Temperamental bulls. Heart rate in Calm bulls remained constant during the study ($P > 0.05$), but fluctuated throughout the study in Temperamental bulls ($P < 0.05$). Cortisol and epinephrine were greater in Temperamental bulls than Calm bulls ($P < 0.01$). There was also a tendency for a temperament by time interaction ($P = 0.07$). Concentrations of norepinephrine were not affected by transportation ($P > 0.05$) or by temperament ($P > 0.05$; data not shown).

Figure 13. Plasma cortisol (A.) and epinephrine (B.) concentrations prior to and during transportation of Brahman bulls for 4 hr (n= 7 Calm and 8 Temperamental bulls).

Cortisol concentrations increased in Calm bulls in response to transportation ($P < 0.05$) but not in Temperamental bulls ($P > 0.05$). Cortisol concentrations were greater in Temperamental bulls than Calm bulls ($P < 0.05$). Epinephrine concentrations were not affected by transportation ($P > 0.05$), but were greater in Temperamental bulls compared to Calm bulls ($P < 0.05$).



epinephrine were greater in Temperamental bulls than Calm bulls ($P < 0.01$). There was also a tendency for a temperament by time interaction ($P = 0.07$). Concentrations of norepinephrine were not affected by transportation ($P > 0.05$) or by temperament ($P > 0.05$; data not shown).

PBMC Proliferation, IgM Production and Gene Expression Analysis

Both proliferation (Figure 14A) and IgM (Figure 14B) production increased dose-dependently in response to treatment with ConA ($P < 0.001$), with neither being affected by temperament ($P > 0.05$). There was a tendency for proliferation to be affected by transportation, with proliferation of PBMCs isolated post-transportation being greater than pre-transportation ($P = 0.056$). The production of IgM by isolated PBMCs was not affected by transportation ($P = 0.28$), although IgM production was numerically greater by PBMCs isolated pre-transportation than post-transportation at the 5 and 10 $\mu\text{g/mL}$ ConA doses.

To determine if transportation influenced the expression of immune- and endocrine-related genes, RNA was extracted from PBMCs pre- and post-transportation. The glucocorticoid receptor (GR) was, and toll-like receptor 4 (TLR-4) tended, to be down-regulated in response to transportation ($P = 0.02$ and $P = 0.08$, respectively; Table 3). The expression of the other genes, interleukin 2 (IL-2), IL-6, IL-10, tumor necrosis factor- α (TNF- α), and TNF- α receptor 1 (TNF- α R1) were not affected by transportation ($P > 0.05$).

Figure 14. Proliferation (A.) and IgM production (B.) of isolated PBMCs pre- and post-transportation of Brahman bulls for 4 hr (n=7 Calm and 8 Temperamental bulls). Means with unlike letters differ ($P < 0.05$). Proliferation and IgM production increased dose-dependently in response to ConA stimulation ($P < 0.05$). However, proliferation was not affected by transportation ($P > 0.05$). Concentrations of IgM tended to decrease in response to transportation ($P < 0.10$).

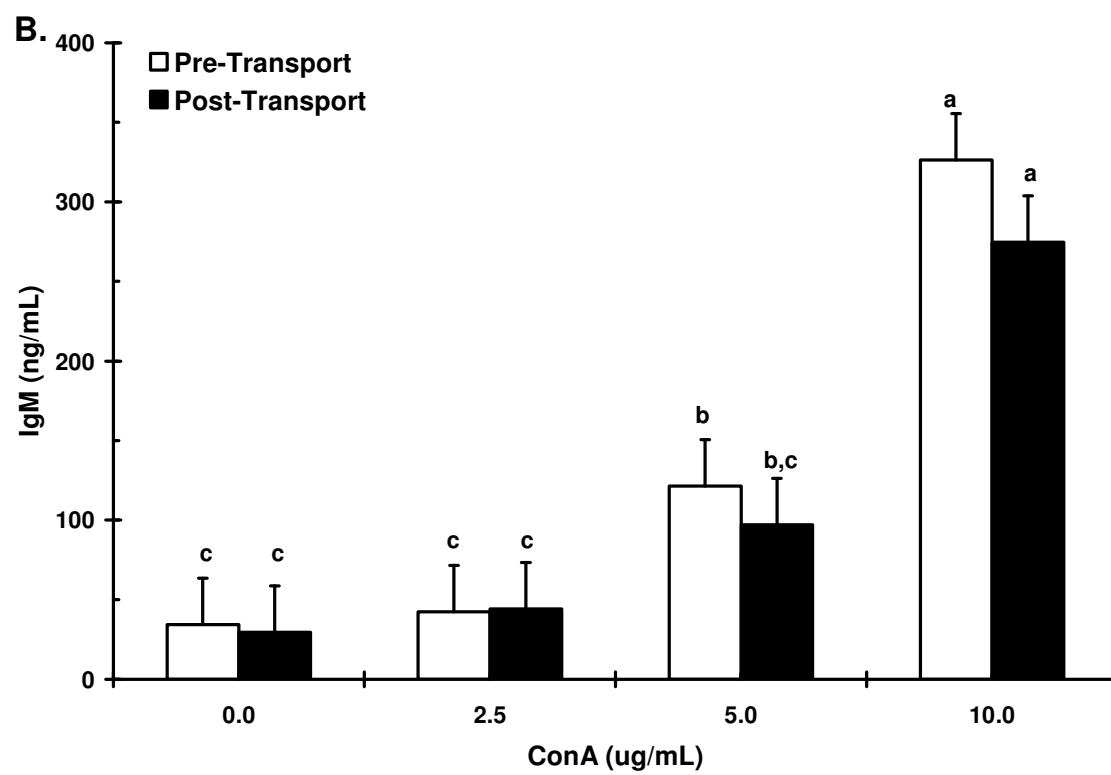
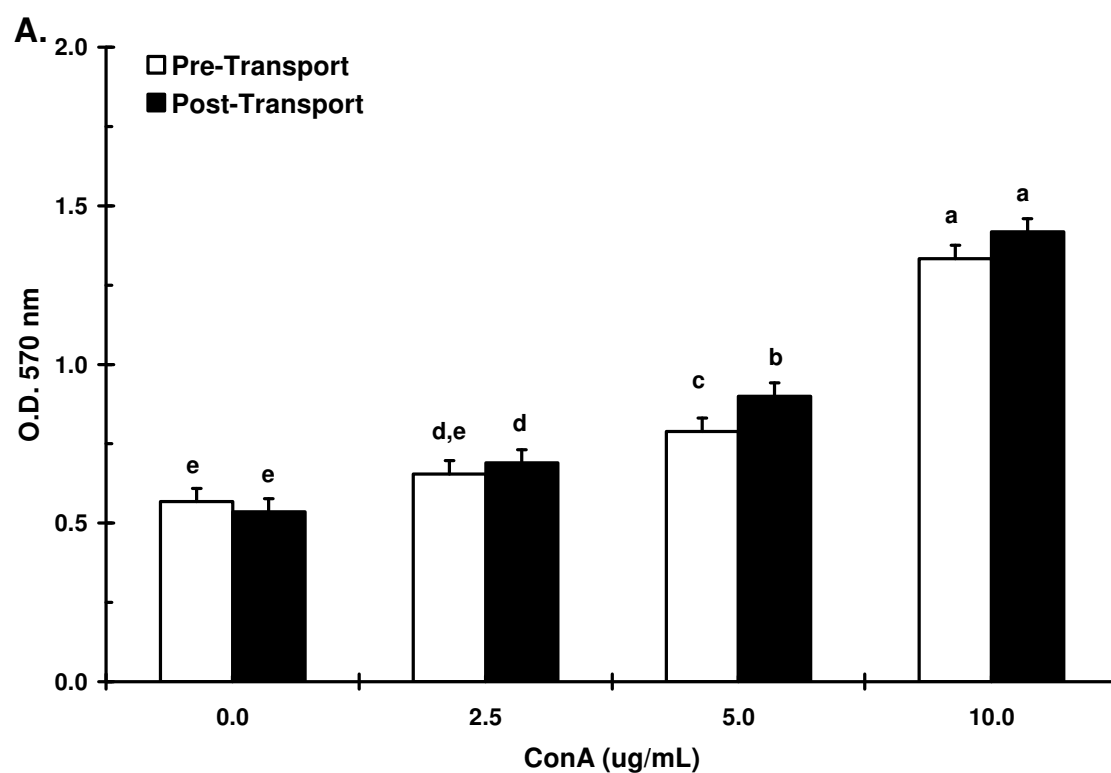


Table 3. Fold change in gene expression of immune- and endocrine-related genes in peripheral blood mononuclear cells (PBMCs) pre- versus post-transportation^{1,2}.

	Fold Change	P-value
GR	0.63 ± 0.10^3	0.02
IL-2	1.53 ± 0.31	0.25
IL-6	1.15 ± 0.24	0.67
IL-10	0.85 ± 0.15	0.51
TNF-α	0.77 ± 0.12	0.18
TNF-α Receptor 1	1.30 ± 0.21	0.33
TLR4	0.76 ± 0.09	0.08

¹Time between collection of pre- and post-transportation samples approximately 6 hours

²Abbreviations: Glucocorticoid receptor (GR); Interleukin-2 (IL-2); Interleukin-6 (IL-6); Interleukin-10 (IL-10); Tumor necrosis factor- α (TNF- α); Tumor necrosis factor- α receptor 1 (TNF- α R1); Toll-like receptor 4 (TLR4)

³Fold change in gene expression relative to 1. Values less than 1 reflect fold decreases in gene expression.

DISCUSSION

The use of remote sampling devices (rectal temperature recording devices, heart rate monitors, and IceSampler™ devices) enabled the authors to monitor specific physiologic and endocrine indices that purportedly reflect transportation-induced stress in cattle. The resultant data indicate that transportation only affected cortisol concentrations in Calm bulls. Additionally, temperament influenced cortisol and epinephrine concentrations as well as heart rate. However, there was no influence of temperament, and limited influence of transportation, on proliferation, IgM production, or cytokine and GR gene expression by isolated PBMCs.

Rectal temperature increased throughout the study and was not affected by transportation or temperament. This is in contrast with our previous report that rectal temperature increased during the first 30 min of transportation and was influenced by temperament (Burdick et al., 2010). However, in our previous study the cattle were not allowed to rest after being loaded onto a trailer and before the initiation of transportation. Therefore, the initial increase in rectal temperature during transportation previously reported may be associated more with the process of loading the cattle into the trailer than solely with the act of transportation.

Temperament differentially affected the cortisol and epinephrine response, but not the norepinephrine response, to transportation. Temperamental bulls may have not been affected by transportation due to their high cortisol and epinephrine concentrations prior to the initiation of transport. A greater basal concentration of cortisol and epinephrine in Temperamental bulls has been previously demonstrated and is

characteristic of Temperamental cattle (Curley et al., 2006; Burdick et al., 2010). Therefore, the greater basal cortisol and epinephrine concentrations may have masked any response to transportation. Consequently, Calm bulls have lower basal concentrations of stress hormones and a response to transportation was evident. Concentrations of epinephrine in Calm bulls were lower than reported previously for basal (91.6 pg/mL) and post-transportation (219.8 pg/mL) of Red Angus bulls (Rulofson et al, 1988). Additionally, pre-transportation concentrations of epinephrine in Temperamental were similar to post-transportation epinephrine concentrations reported by Rulofson et al. (1988). Concentrations of norepinephrine were not affected by transportation, and were lower than previously reported post-transportation norepinephrine concentrations (321.5 pg/mL; Rulofson et al., 1988). The lack of effect of transportation on norepinephrine concentrations is in contrast to a report by Odore et al. (2004) who transported Blonde D'Aquitaine calves for 14 hr and found that norepinephrine, but not epinephrine, increased in response to transportation. As discussed previously, this difference may be due to the influence of loading and unloading the cattle, as post-transportation samples were collected following unloading in contrast to the current study in which all samples were collected while bulls remained on the trailer. This has also been stated by others, including Odore et al. (2004), that the handling and unloading appeared more stressful on calves than the actual transport. Although some changes in hormone secretion were attributable to handling and temperament, transportation did not result in similar responses between temperament

groups. Future research needs to elucidate the potential influence of temperament on stress responses to loading, transporting and unloading beef cattle.

In response to an infection there is an increase in the production and secretion of cytokines, as well as changes in various immune and endocrine receptors. The pro-inflammatory cytokines, TNF- α , IL-2, and IL-6 are involved in the initial innate immune system response and have roles in the activation of the adaptive immune system. The TNF- α R1 is the main receptor for TNF- α and elicits responses in a variety of tissues, including signaling of apoptosis of immune cells *in vitro* (Pfeffer, 2003). However, there was no change in the expression of these cytokines due to transportation or temperament. Toll-like receptor 4 is a receptor that allows for the recognition of gram negative bacteria, specifically lipopolysaccharide. The tendency for a down-regulation of TLR4 in response to transportation may leave the animal more susceptible to infection by gram negative bacteria after their arrival at their destination following transportation.

The GR mediates the actions of cortisol in tissues, including cells of the immune system. It is found in almost all body tissues and is regulated by tissue-specific mechanisms (De Bosscher and Hageman, 2009). The GR can regulate transcription of genes through several mechanisms including binding directly to glucocorticoid response elements (GRE) on DNA, through protein-protein interactions with transcription factors, and by influencing mRNA degradation. A small percentage of genes are directly regulated by GR through a conventional GRE, with many subjected to other regulatory mechanisms (De Bosscher and Hageman, 2009). Transrepression of genes by the GR is mainly mediated through protein-protein interactions that inhibit the transcription factors

nuclear factor- κ B (NF- κ B) and activating protein-1 (AP-1). Immune related genes regulated in this manner include IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-8, IL-12, IL-18, interferon- γ (IFN- γ), TNF- α , as well as other growth factors (Ashwell et al., 2000; De Bosscher and Hageman, 2009). All of these genes have one or more NF- κ B or AP-1 binding sites in their promoter region (De Bosscher and Hageman, 2009). Additionally, the GR inhibits stability of mRNA encoding certain cytokines (Ashwell et al., 2000). Overall, the modulation of gene expression by the GR can affect apoptosis, adhesion, inflammation, and basic functions of immune system components (Buckham Sporer et al., 2008). Therefore, actions of glucocorticoids elicited through the binding to GR can significantly alter immune responses through negatively regulating immune activation and communication.

In response to transportation, it was not unexpected to find that the GR is down-regulated, as cortisol is known to down-regulate the GR in response to excessive cortisol concentrations (Gross et al., 2009). A decrease in expression of GR is in agreement with a study that found a decrease in the concentrations of both GR and the β -adrenergic receptor, the receptor for epinephrine and norepinephrine, following a 14-hr transportation (Odore et al., 2004). However, there was no change in the expression of GR in isolated neutrophils following 9.75 hr of transportation in Belgian Blue x Friesian bulls (Buckham Sporer et al., 2007). A decrease in the expression of the GR may result in a period of glucocorticoid insensitivity. As binding of cortisol to the GR results in inhibition of the inflammatory response (pro-inflammatory cytokines), a decrease in the

GR may result in excessive inflammation if the anti-inflammatory signal from the GR does not occur.

Stress hormones produced in response to stressors have been demonstrated to inhibit adaptive immune functions (Blecha et al., 1984; Rinner et al., 1992; Bauer et al., 2001). The ability of isolated lymphocytes to proliferate and produce immunoglobulin in response to a mitogen is often used to measure the cell-mediated and humoral adaptive immune responses, respectively (Bauer et al., 2001). The mitogen ConA is a plant lectin that is used to activate T cells, resulting in proliferation. Activated T cells then stimulate B cells, resulting in an increase in production of immunoglobulins, primarily IgM. The current study found no change in the proliferation of isolated PBMCs immediately following transportation or due to temperament. In contrast, Blecha et al. (1984) found a decrease in ConA-induced proliferation at unloading of Angus and Angus x Brahman steers following a 10-hr transport. Murata et al. (1985) in which castrated Holstein calves were transported for 1 hr found an increase in lymphocyte proliferation to phytohemmagglutinin-P (PHA-P), ConA, and pokeweed Mitogen (PWM) 6-hr after transport, with a recovery to basal proliferation values within 24 hr. The authors also found a decrease in cortisol concentrations at this time. A later study by the same authors found that a 4-hr transport of Holstein calves decreased basal lymphocyte proliferation in response to PHA (Murata et al., 1987). Therefore, differences in the proliferative ability of calves may be due to the length of transportation as well as the mitogen used to induce proliferation. Unfortunately, there are limited publications on the effects of transportation on mitogen-induced PBMC IgM

production. A decrease in the ability of isolated lymphocytes to proliferate and to produce IgM may be indicative of an inhibited humoral immune response, thus leaving an animal more susceptible to pathogens.

Stressful events including transportation have been implicated in the increased incidence of disease in cattle upon arrival at stockyards and feedlots, with bovine respiratory disease being the most common (Fike and Spire, 2006; Buckham Sporer et al., 2007, Buckham Sporer et al., 2008). Transportation has been demonstrated to increase the number of circulating neutrophils, and increase their lifespan (Buckham Sporer et al., 2007, 2008). As neutrophils are usually short-lived cells during an immune response, their enhanced lifespan may be detrimental as neutrophils could potentially damage healthy tissue. Although there were no apparent changes in the expression of cytokines or in the proliferative and IgM responses of isolated PBMCs, samples were only obtained immediately following the end of transportation. It is possible that if samples were obtained in the hours following the end of transportation that differences in expression would be evident.

CONCLUSIONS

While transportation has been purported to be a stressor in cattle, our data indicates that the process of loading and unloading cattle may be more stressful than the transportation itself. Transportation only influenced cortisol concentrations in Calm bulls, while temperament influenced cortisol and epinephrine concentrations in addition to heart rate. Further research is needed to elucidate the physiological and

endocrinologic changes in response to the loading and unloading of cattle into a trailer, and whether it is beneficial to rest the cattle on the trailer before commencing transportation. Additionally, the effects of transportation on immune function were limited. This may be due to the fact that samples were not collected at later time points following the end of transportation. Therefore, transportation may have influenced immune function in a manner that may not have been apparent at the end of transportation. Future studies should focus on determining the effect of transportation on gene expression, proliferation, and IgM production hours to days following transportation.

CHAPTER VI

ENDOGENOUS CORTISOL ACUTELY MODULATES CYTOKINE GENE EXPRESSION IN BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS

INTRODUCTION

Late in the 1940s the anti-inflammatory actions of glucocorticoids were discovered (Hench et al., 1949). This puzzled many glucocorticoid physiologists as up until this time it was believed that stress enhanced, not suppressed, immune defense mechanisms (Munck et al., 1984; Guyre et al., 2008). Since then much literature has been published, and it has become a well-known fact that glucocorticoids are anti-inflammatory and immunosuppressive. For example, glucocorticoids are known to suppress many immune functions including but not limited to suppressing maturation, differentiation, and proliferation of all immune cells, inducing apoptosis of lymphocytes, reducing chemotaxis of leukocytes, and decreasing the number of circulating leukocytes (Martin, 2009). However, it should not be astonishing that stress inhibits the immune system, as the stress response redirects resources to processes essential to the immediate survival of the organism. Additionally, down-regulation of the immune system during periods of stress minimizes the potential for damage by hyperactive immune cells (Sapolsky et al., 2000; Martin, 2009). However, recently this concept has been challenged due to the fact that a functional immune system is necessary if an animal must defend itself from an immune challenge following recovery from a stressor.

Recent publications suggest acute exposure to glucocorticoids may actually enhance immune function (Dhabhar et al. 2009; Martin, 2009). These positive effects of stress include redistribution of immune cells to enhance immune function in organs (e.g., the skin), and may promote wound healing and elimination of infection (Dhabhar, 2000; Dhabhar, 2002; Dhabhar et al., 2009). However, most of the studies seeking to elucidate these mechanisms utilize rodent models which may not be appropriate for humans or larger livestock species such as cattle due to the use of inbred models and genetic selection in rodents. Therefore, the objective of this study was to determine the influence of acute endogenous increases in cortisol on cytokine and glucocorticoid receptor (GR) gene expression in peripheral blood mononuclear cells in response to 1) cannulation and 2) adrenocorticotrophic hormone (ACTH) challenge in cattle.

MATERIALS AND METHODS

Experimental Design

All experimental procedures were in compliance with the Guide for the Care and Use of Agricultural Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee at Texas A&M University. Brahman heifers (n = 12; 334 ± 12 kg BW) were utilized for the study. Indwelling jugular catheters were inserted non-surgically at the onset of the study (0.5 to 1.25 hr prior to collection of first sample). During these procedures cattle were restrained in a working chute for approximately 5 to 10 min. Following cannulation heifers were moved into individual stanchions for the remainder of the study. For determination of cortisol concentrations whole blood

samples were collected and transferred into heparinized vacutainers beginning -3 hr and continuing every 0.25 hr until 4 hr post challenge with ACTH (0.1 IU/kg BW i.v.). Blood samples were processed immediately by centrifugation at 1500 x g for 10 min at 4°C. Isolated plasma samples were stored at -80°C until analysis for cortisol concentrations. For isolation of PBMCs whole blood samples were collected and transferred to vacutainers containing EDTA at cannulation, 0, 1, 2 and 4 hrs relative to ACTH challenge, and processed as described below.

PBMC Isolation, RNA Extraction and Quantitative Real Time RT-PCR Analysis

The PBMCs were isolated using density gradient centrifugation using Ficoll-Paque Plus (Cat. No. 17-1440-03; VWR, West Chester, PA, USA). Erythrocytes were lysed using a 0.2% NaCl solution followed by the addition of 1.6% NaCl solution to achieve 0.9% salinity. Isolated PBMCs were resuspended in DME/F12 and frozen at -80°C until RNA extraction.

Total RNA was extracted from PBMCs using Invitrogen's PureLink Micro-to-Midi RNA Purification System according to the manufacturer's instructions (Cat. No. 12183-018; Invitrogen, Carlsbad, CA, USA). Quality and quantity of RNA in elutes were determined using a spectrophotometer. Extracted RNA was treated with DNase I and cDNA synthesis was performed (all reagents from Invitrogen). Bovine-specific primers were designed using Primer Express Software (Table 4; Applied Biosystems, Foster City, CA, USA) and were ordered from Sigma Genosys. Reactions containing 20

Table 4. Bovine primers for quantitative real-time RT-PCR analysis for determination of changes in gene expression in response to cannulation and adrenocorticotropin (ACTH) challenge^{1,2}.

	Forward Primer 5'→3'	Reverse Primer 5'→3'
IL-10	GCCTTGTCGGAAATGATCCA	TCAGGCCCGTGGTTCTCA
IFN-γ	TCTGCAGATCCAGCGCAAA	CGGCCTCGAAAGAGATTCTGA
IL-4	ACGCTGAACATCCTCACAACG	AGCTCAATTCCAACCCTGCAG
TNF-α	CCGGTGGTGGGACTCGTAT	GCTGGTTGTCTTACAGCTTCACA
GR	TCAACTTGGCGGATCATGAC	CATTTCACGGCTGCAATCAC
18s	GTAACCCGTTGAACCCCAT	CCATCCAATCGGTAGTAGCG

¹Primers were designed using Primer Express Software (Applied Biosystems, Foster City, CA) and were ordered from Sigma Genosys.

²Abbreviations: Interleukin-10 (IL-10); Interferon- γ (IFN- γ); Interleukin-4 (IL-4); Tumor necrosis factor- α (TNF- α); Glucocorticoid receptor (GR); 18s rRNA (18s)

ng of cDNA were amplified in triplicate 25 μ L reactions containing 2x SYBR Green PCR Mastermix (Applied Biosystems) and 6.25 nM of each primer. The 7900HT Fast Real-Time PCR System (Applied Biosystems) was used with the following conditions: heat inactivation at 50°C for 15 s, denaturation at 95°C for 10 min and annealing/extension at 56°C for 1 min for 40 cycles, and dissociation curves obtained from 60°C to 95°C.

Cortisol Concentrations

Serum concentrations of cortisol were determined using a single antibody radioimmunoassay (DSL-2100; Diagnostic Systems Labs, Webster, TX) utilizing rabbit anti-cortisol antiserum coated tubes according to the manufacturer's directions (Burdick et al., 2009a). The minimum detectable cortisol concentration was 1.2 ng/mL and the intra- and inter-assay coefficients of variation were 7.7% and 7.6%, respectively. Serum concentrations of cortisol were determined by comparison to a standard curve generated with known concentrations of cortisol and presented as the concentration in ng/mL.

Statistical Analysis

All PCR reactions were normalized to bovine 18s rRNA by dividing the Ct values for the gene of interest by the Ct value for 18s rRNA. Relative differences were then calculated using the $2^{-\Delta\Delta C_T}$ method (Livack and Schmittgen, 2001) to determine fold change in gene expression compared to either cannulation (-3 hrs) or time 0 (prior to

administration of ACTH). There was no difference in expression of 18s rRNA over time.

All data were analyzed first using the MIXED procedure of SAS (SAS Inst. Inc., Cary, NC, USA) specific for repeated measures with time included as the repeated effect and heifer included as the subject. All data were analyzed again using the MIXED procedure of SAS specific for repeated measures with time included as the repeated effect, heifer included as the subject, and time between cannulation and collection of the first sample included as a covariate. Data are presented as least square means \pm SEM with a P -value of < 0.05 considered significant.

RESULTS

Cannulation Data Analyzed Without Covariate

To determine if the cannulation procedure increased cortisol and subsequently gene expression in isolated PBMCs, whole blood was collected every 0.5 hr beginning after the cannulation procedure (- 3 hr) and immediately prior to the ACTH challenges (time 0). Cortisol concentrations tended to decrease ($P = 0.07$; Fig. 15) from cannulation (-3 hrs) to time 0. Additionally, gene expression of all genes of interest tended to increase compared to gene expression at cannulation (Table 5; $P = 0.06$ -0.14), with IFN- γ displaying a 16.43 ± 4.39 -fold increase in gene expression ($P = 0.05$).

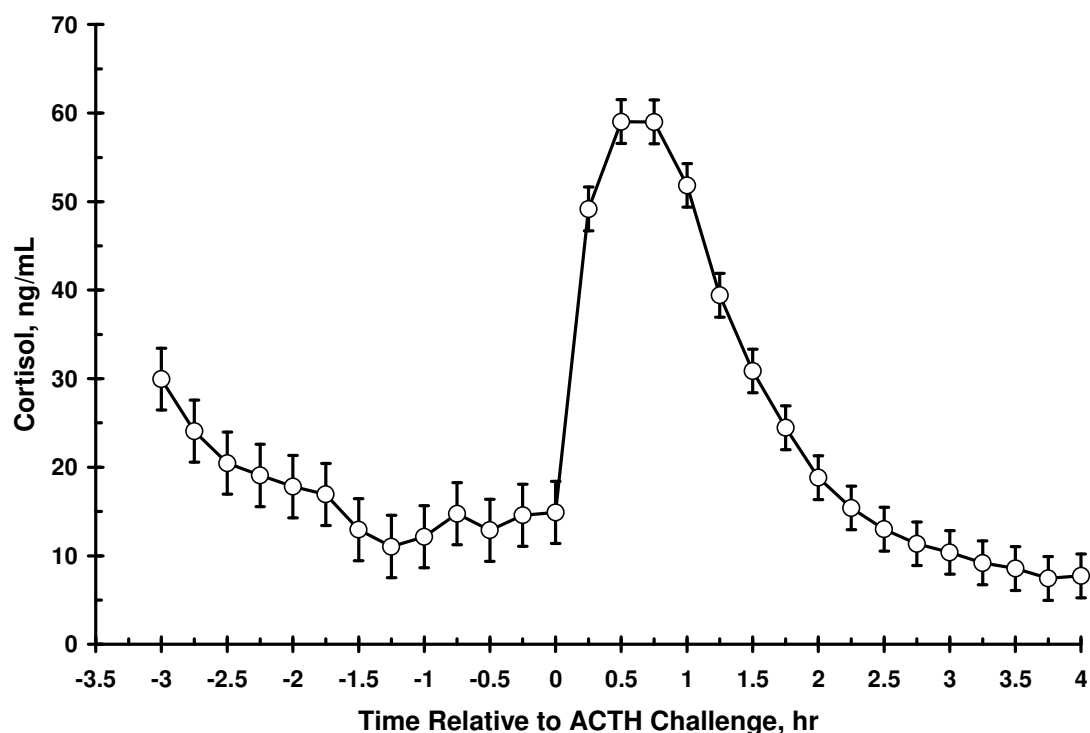


Figure 15. Plasma cortisol concentrations of Brahman heifers in response to cannulation and adrenocorticotropin (ACTH) challenge with data analyzed without the addition of a covariate. Whole blood was collected and plasma isolated beginning at cannulation (-3 hr) prior to an adrenocorticotrophic hormone (ACTH) challenge (time 0), and continued at 15 min intervals through 4 hr post challenge. Cortisol concentrations decreased from cannulation through time 0 ($P = 0.01$). In response to ACTH challenge cortisol concentrations increased, peaking at 30 min ($P < 0.001$).

Table 5. Fold change in gene expression of immune- and endocrine-related genes at the onset of adrenocorticotropin (ACTH) challenge (time 0) relative to gene expression at cannulation analyzed without a covariate^{1,2}.

	Fold Change	<i>P</i>-value
IL-10 ²	4.41 ± 1.26	0.09
IFN-γ ³	16.43 ± 4.52	0.04
IL-4 ⁴	8.37 ± 2.60	0.08
TNF-α ⁵	67.79 ± 28.85	0.14
GR ⁶	30.85 ± 9.90	0.06

¹Time between cannulation and time 0 approximately 3 hr

²Abbreviations: Interleukin-10 (IL-10); Interferon- γ (IFN- γ); Interleukin-4 (IL-4); Tumor necrosis factor- α (TNF- α); Glucocorticoid receptor (GR).

Cannulation Data Analyzed with Covariate

In a subsequent analysis, the time between cannulation and collection of the first sample (ranging from 30 to 45 min) was added as a covariate. With the addition of the covariate, concentrations of cortisol displayed a similar pattern as the previous analysis. Concentrations of cortisol decreased from -3 hr to time 0, prior to the administration of ACTH (Fig. 16; $P = 0.004$), and with variability in cortisol concentrations partially explained by addition of the covariate ($P = 0.001$). Similar to the previous data analysis, gene expression of all genes of interest tended to increase compared to gene expression at cannulation (Table 6; $P = 0.08$ - 0.15), with IFN- γ displaying a 16.69 ± 4.71 -fold increase in gene expression ($P = 0.05$). Addition of the covariate did not explain variation in gene expression for any of the genes of interest ($P = 0.273$ - 0.789).

ACTH Challenge Data Analyzed Without Covariate

An acute ACTH challenge was utilized to determine the influence of acute increases in endogenous cortisol on gene expression in isolated PBMCs. Cortisol concentrations increased in response to ACTH challenge, peaking within 0.5 hr ($P < 0.001$; Fig. 15) before decreasing to pre-challenge values by 2 hr post-challenge. Expression of IL-10 increased 3.97 ± 0.70 fold at 1 hr post-challenge before decreasing ($P = 0.023$; Fig. 17). There was a trend for the expression of IFN- γ to change over time ($P = 0.164$; Fig. 18), with the greatest numerical fold increase in expression occurring 2 hr post-challenge. The greatest fold increase in expression of IL-4 (9.44 ± 1.97 ; Fig. 19) occurred at 4 hr post-challenge ($P = 0.037$). However, it is possible that the greatest fold

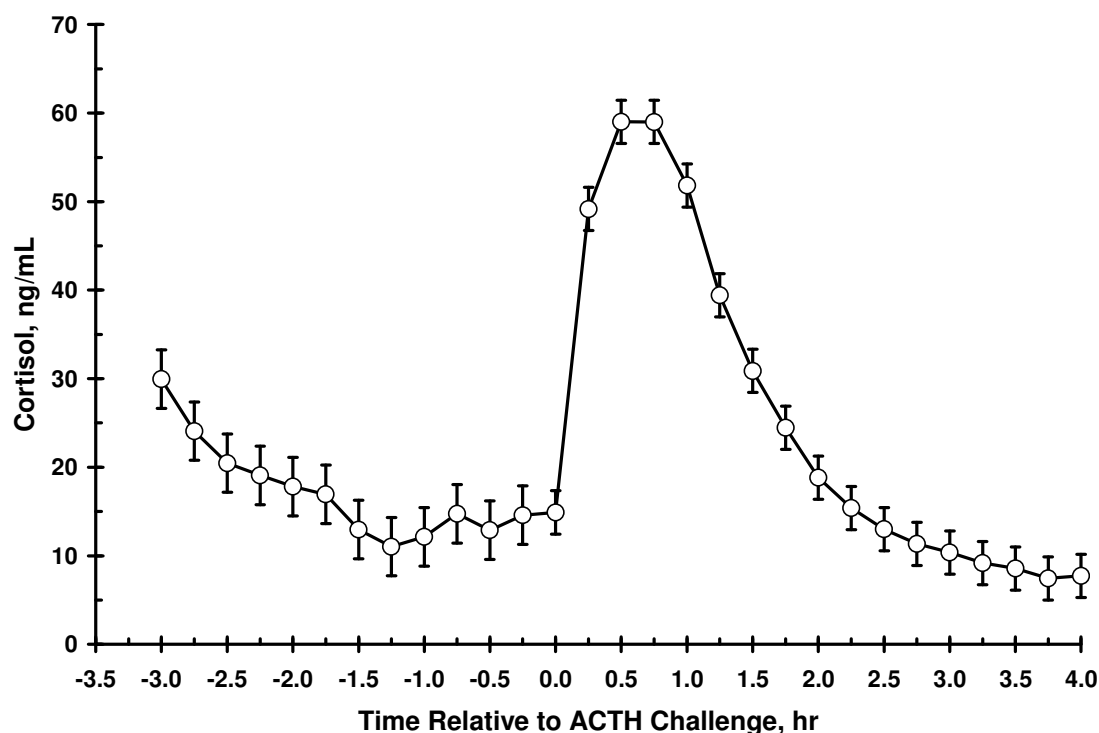


Figure 16. Plasma cortisol concentrations of Brahman heifers in response to cannulation and adrenocorticotropin (ACTH) challenge with data analyzed with the addition of a covariate (time between cannulation and collection of first sample at time 0; 30 to 75 min). Whole blood was collected and plasma isolated beginning at cannulation (-3 hr) prior to an adrenocorticotrophic hormone (ACTH) challenge (time 0), and continued at 15 min intervals through 4 hr post challenge. Cortisol concentrations decreased from cannulation through time 0 ($P = 0.01$) and were affected by time between cannulation and collection of first sample ($P = 0.004$). In response to ACTH challenge cortisol concentrations increased, peaking at 30 min ($P < 0.001$) and were affected by time between cannulation and collection of first sample ($P = 0.028$).

Table 6. Fold change in gene expression of immune- and endocrine-related genes at the onset of adrenocorticotropin (ACTH) challenge (time 0) relative to gene expression at cannulation analyzed with a covariate^{1,2,3}.

	Fold Change	Time <i>P</i>-value	Covariate <i>P</i>-value
IL-10	4.51 ± 1.27	0.08	0.36
IFN-γ	16.69 ± 4.71	0.05	0.70
IL-4	8.37 ± 2.68	0.09	0.72
TNF-α	67.79 ± 29.72	0.15	0.79
GR	30.74 ± 9.77	0.06	0.27

¹Time between cannulation and time 0 approximately 3 hr.

²Covariate: time between cannulation and collection of first sample (0.5 to 1.25 hr).

³Abbreviations: Interleukin-10 (IL-10); Interferon- γ (IFN- γ); Interleukin-4 (IL-4); Tumor necrosis factor- α (TNF- α); Glucocorticoid receptor (GR).

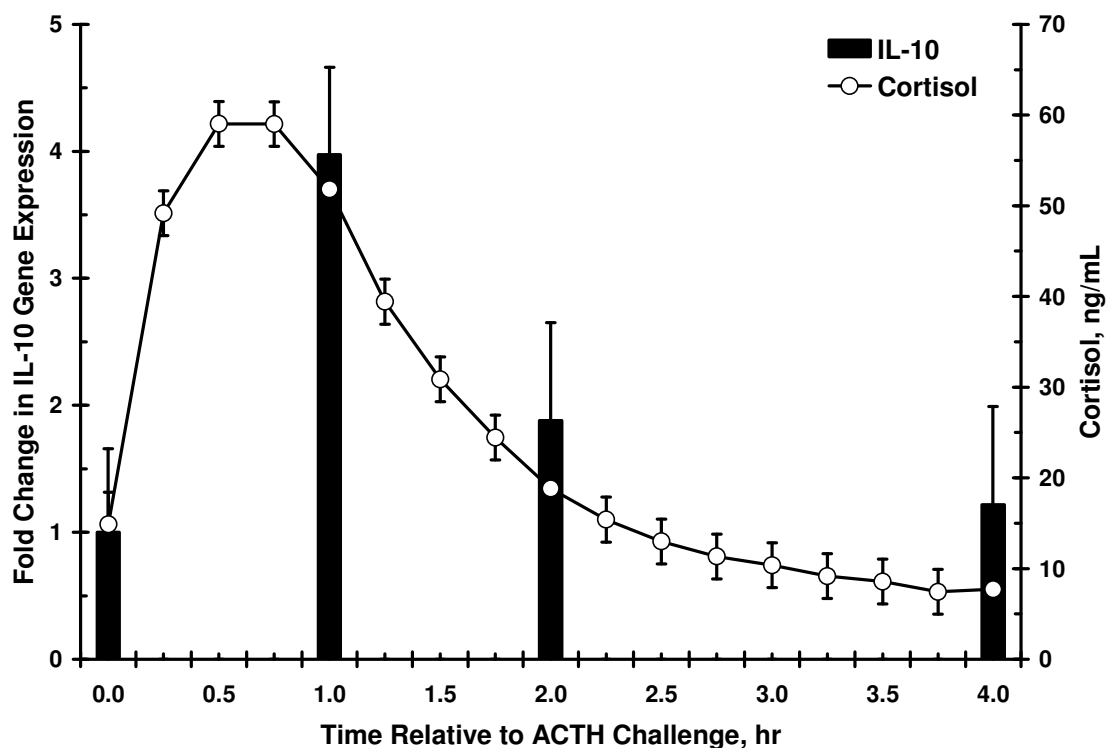


Figure 17. Fold change in gene expression of IL-10 in isolated peripheral blood mononuclear cells (PBMCs) in response to adrenocorticotropin (ACTH) challenge with data analyzed without the addition of a covariate. Data plotted in reference to cortisol concentrations post-ACTH challenge. Whole blood was collected and PBMCs isolated at 0, 1, 2 and 4 hr relative to a challenge with 0.1 IU/kg BW ACTH. Total RNA was extracted from isolated PBMCs and gene expression determined using quantitative real-time RT-PCR. Expression of IL-10 increased 1 hr post-ACTH challenge before decreasing ($P = 0.023$; LS Mean \pm SEM).

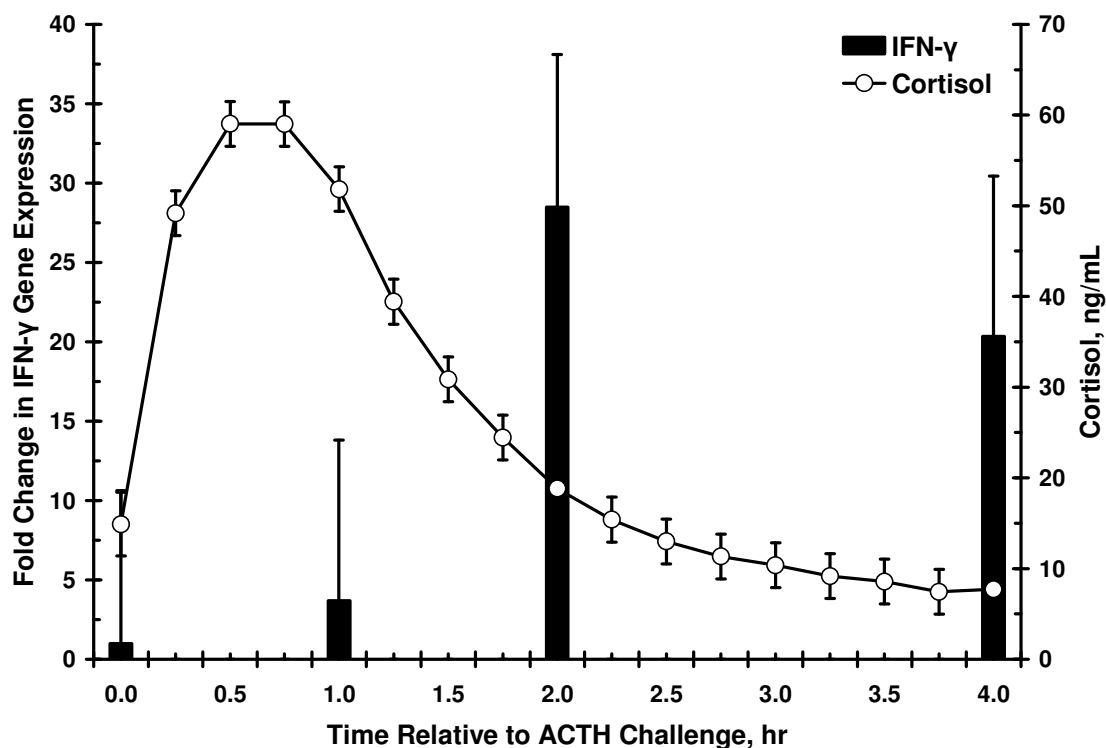


Figure 18. Fold change in gene expression of IFN- γ in isolated peripheral blood mononuclear cells (PBMCs) in response to adrenocorticotropin (ACTH) challenge with data analyzed without the addition of a covariate. Data plotted in reference to cortisol concentrations post-ACTH challenge. Whole blood was collected and PBMCs isolated at 0, 1, 2 and 4 hr relative to a challenge with 0.1 IU/kg BW ACTH. Total RNA was extracted from isolated PBMCs and gene expression determined using quantitative real-time RT-PCR. There was a trend for the expression of IFN- γ to increase over time, with the greatest numerical fold increase occurring 2 hr post-challenge ($P = 0.16$; LS Mean \pm SEM).

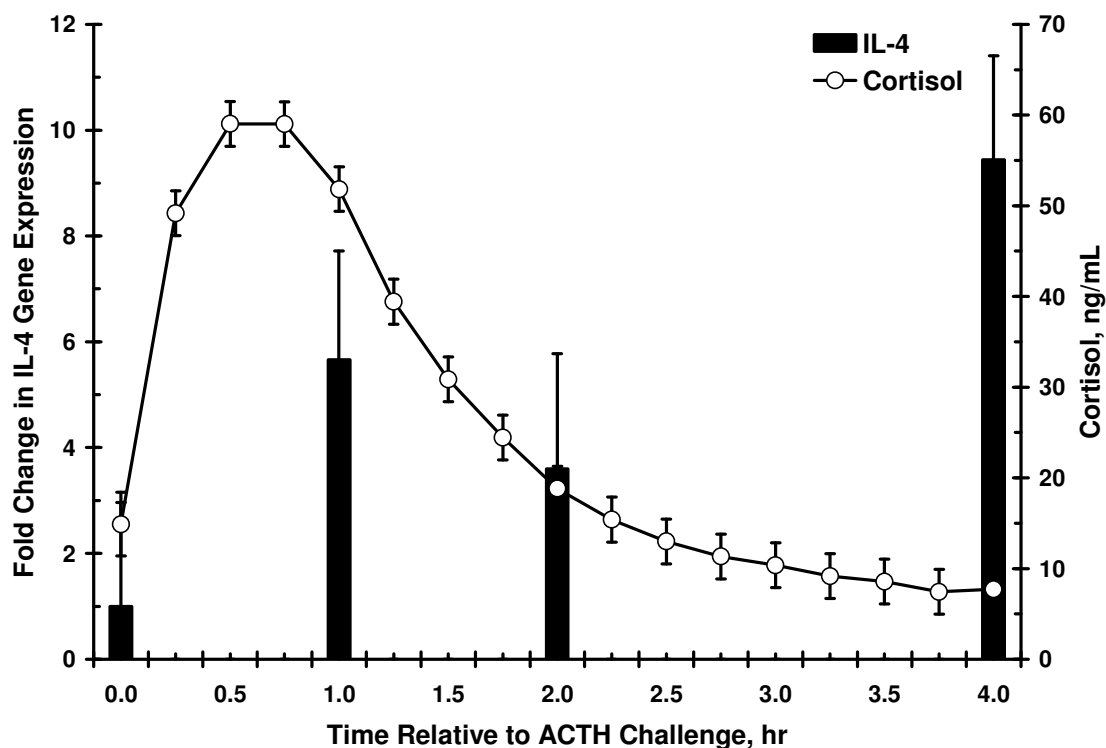


Figure 19. Fold change in gene expression of IL-4 in isolated peripheral blood mononuclear cells (PBMCs) in response to adrenocorticotropin (ACTH) challenge with data analyzed without the addition of a covariate. Data plotted in reference to cortisol concentrations post-ACTH challenge. Whole blood was collected and PBMCs isolated at 0, 1, 2 and 4 hr relative to a challenge with 0.1 IU/kg BW ACTH. Total RNA was extracted from isolated PBMCs and gene expression determined using quantitative real-time RT-PCR. The greatest fold increase in expression of IL-4 occurred 4 hr post-challenge ($P = 0.037$; LS Mean \pm SEM).

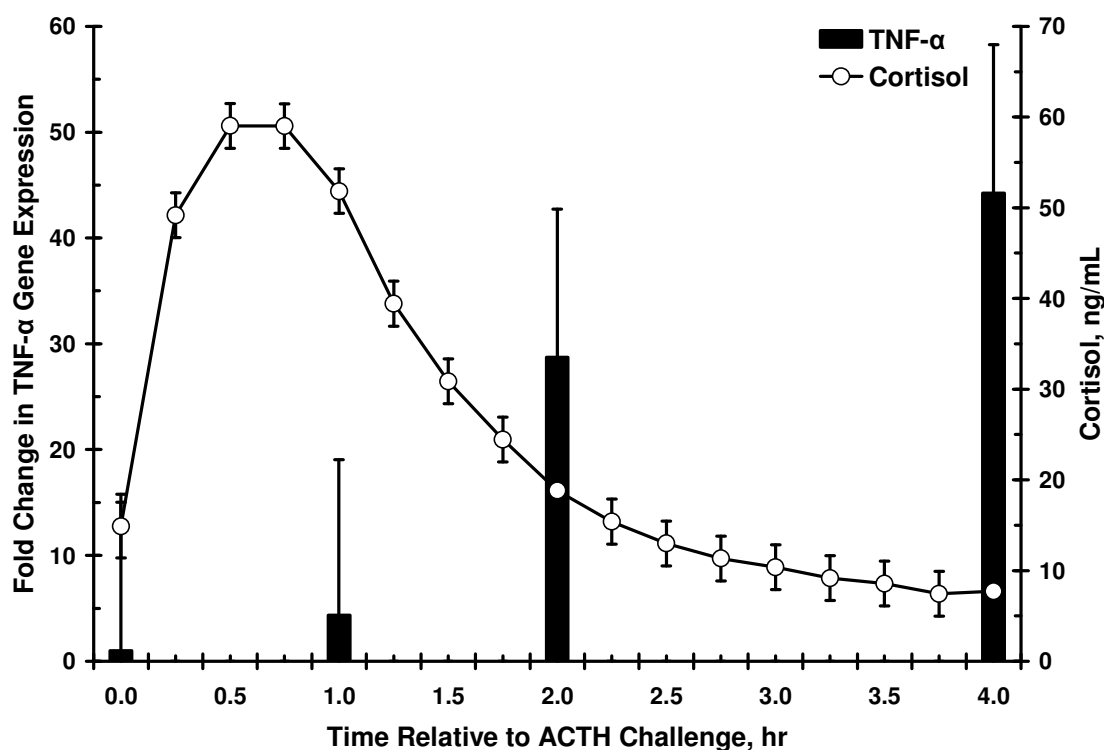


Figure 20. Fold change in gene expression of TNF- α in isolated peripheral blood mononuclear cells (PBMCs) in response to adrenocorticotropin (ACTH) challenge with data analyzed without the addition of a covariate. Data plotted in reference to cortisol concentrations post-ACTH challenge. Whole blood was collected and PBMCs isolated at 0, 1, 2 and 4 hr relative to a challenge with 0.1 IU/kg BW ACTH. Total RNA was extracted from isolated PBMCs and gene expression determined using quantitative real-time RT-PCR. The gene expression of TNF- α tended to increase, with the greatest numerical fold increase in expression of occurred 4 hr post-challenge ($P = 0.120$; LS Mean \pm SEM).

change in expression occurred later than 4 hr post-challenge, as later samples were not collected. The expression of TNF- α tended to increase in response to ACTH challenge ($P = 0.120$; Figure 20). In contrast, the expression of the GR did not change in response to ACTH challenge ($P = 0.256$; Figure 21).

ACTH Challenge with Covariate Addition

Data were reanalyzed with the time between cannulation and collection of the first sample (ranging from 0.5 to 1.25 hr) included as a covariate. Similar results were found for all variables analyzed. Cortisol concentrations increased in response to ACTH challenge, peaking within 0.5 hr ($P < 0.001$; Fig. 16) before decreasing to pre-challenge values by 2 hr post-challenge. Variation in post-challenge cortisol concentrations can be partially explained by the covariate ($P = 0.028$). Expression of IL-10 increased 3.97 ± 0.70 fold at 1 hr post-challenge before decreasing ($P = 0.020$; Fig. 22). There was a trend for the expression of IFN- γ to change over time ($P = 0.170$; Fig. 23), with the greatest numerical fold increase in expression occurring 2 hr post-challenge. The greatest fold increase in expression of IL-4 (9.26 ± 1.86 ; Fig. 24) occurred at 4 hr post-challenge ($P = 0.032$). However, it is possible that the greatest fold change in expression occurred later than 4 hr post-challenge, as later samples were not collected. The expression of TNF- α tended to increase in response to ACTH challenge ($P = 0.121$; Figure 25). In contrast, the expression of the GR did not change in response to ACTH challenge ($P = 0.270$; Figure 26). The addition of the covariate partially explained

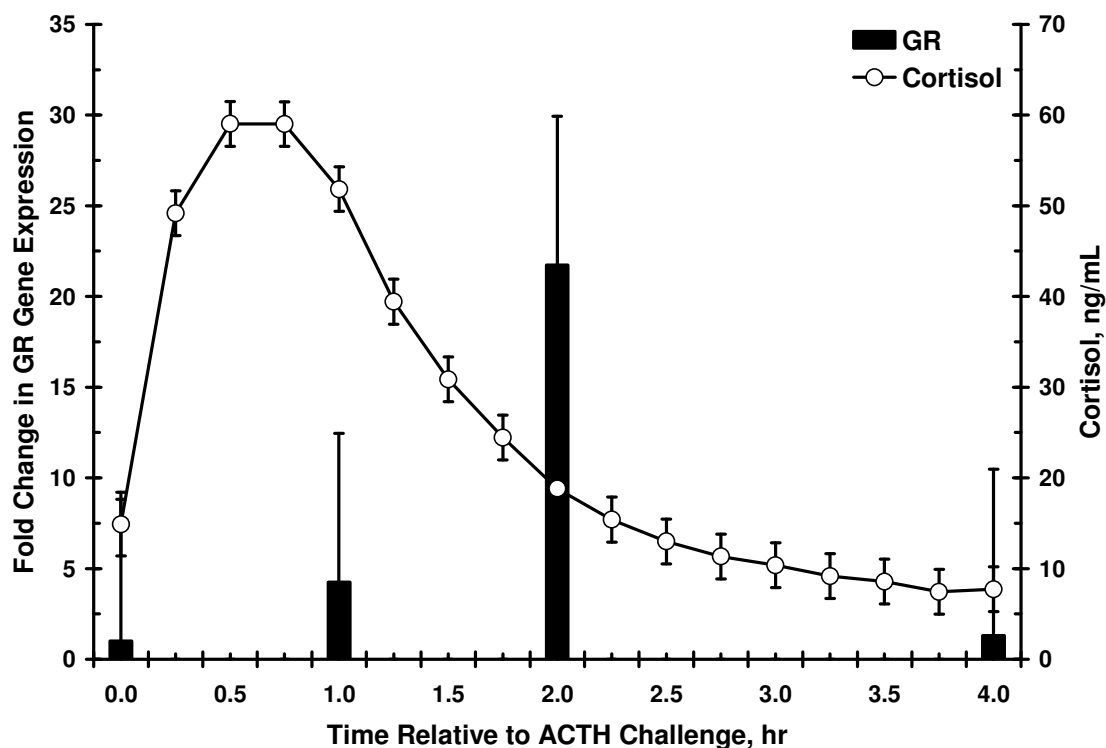


Figure 21. Fold change in gene expression of GR in isolated peripheral blood mononuclear cells (PBMCs) in response to adrenocorticotropin (ACTH) challenge, with data analyzed without the addition of a covariate. Data plotted in reference to cortisol concentrations post-ACTH challenge. Whole blood was collected and PBMCs isolated at 0, 1, 2 and 4 hr relative to a challenge with 0.1 IU/kg BW ACTH. Total RNA was extracted from isolated PBMCs and gene expression determined using quantitative real-time RT-PCR. The gene expression of GR did not change in response to ACTH challenge ($P = 0.256$; LS Mean \pm SEM).

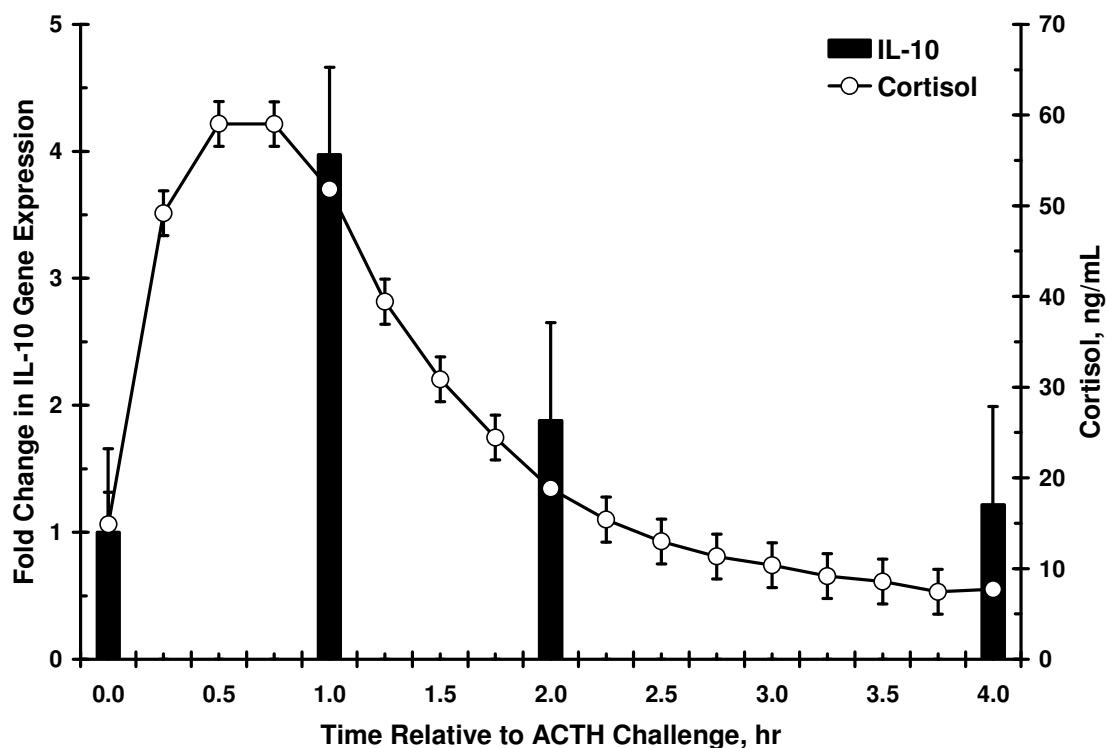


Figure 22. Fold change in gene expression of IL-10 in isolated peripheral blood mononuclear cells (PBMCs) in response to adrenocorticotropin (ACTH) challenge with data analyzed with the addition of a covariate. The covariate included in the data analysis was the time between cannulation and collection of the first sample (ranging from 0.5 to 1.25 hr). Data plotted in reference to cortisol concentrations post-ACTH challenge. Whole blood was collected and PBMCs isolated at 0, 1, 2 and 4 hr relative to a challenge with 0.1 IU/kg BW ACTH. Total RNA was extracted from isolated PBMCs and gene expression determined using quantitative real-time RT-PCR. Expression of IL-10 increased 1 hr post-ACTH challenge before decreasing ($P = 0.020$; LS Mean \pm SEM). The covariate explained some variation associated with the expression of IL-10 ($P = 0.052$).

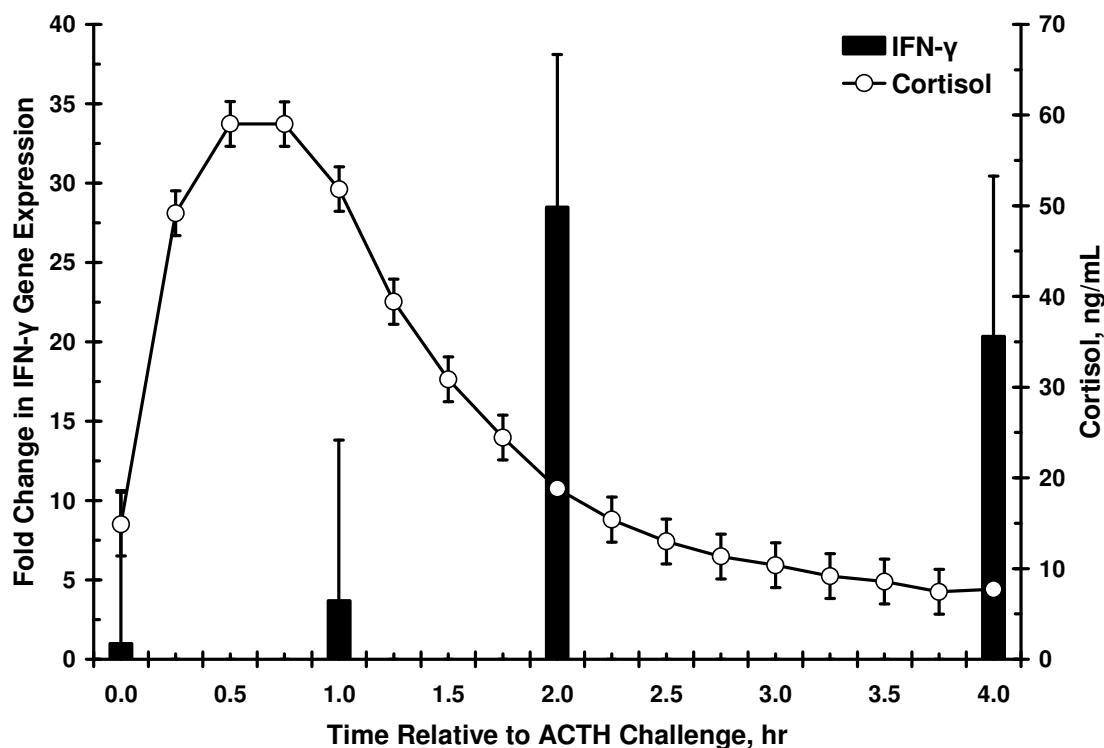


Figure 23. Fold change in gene expression of IFN- γ in isolated peripheral blood mononuclear cells (PBMCs) in response to adrenocorticotropin (ACTH) challenge with data analyzed with the addition of a covariate. The covariate included in the data analysis was the time between cannulation and collection of the first sample (ranging from 0.5 to 1.25 hr). Data plotted in reference to cortisol concentrations post-ACTH challenge. Whole blood was collected and PBMCs isolated at 0, 1, 2 and 4 hr relative to a challenge with 0.1 IU/kg BW ACTH. Total RNA was extracted from isolated PBMCs and gene expression determined using quantitative real-time RT-PCR. There was a trend for the expression of IFN- γ to increase over time, with the greatest numerical fold increase occurring 2 hr post-challenge ($P = 0.170$; LS Mean \pm SEM). The covariate did not explain variation in the expression of IFN- γ ($P = 0.402$).

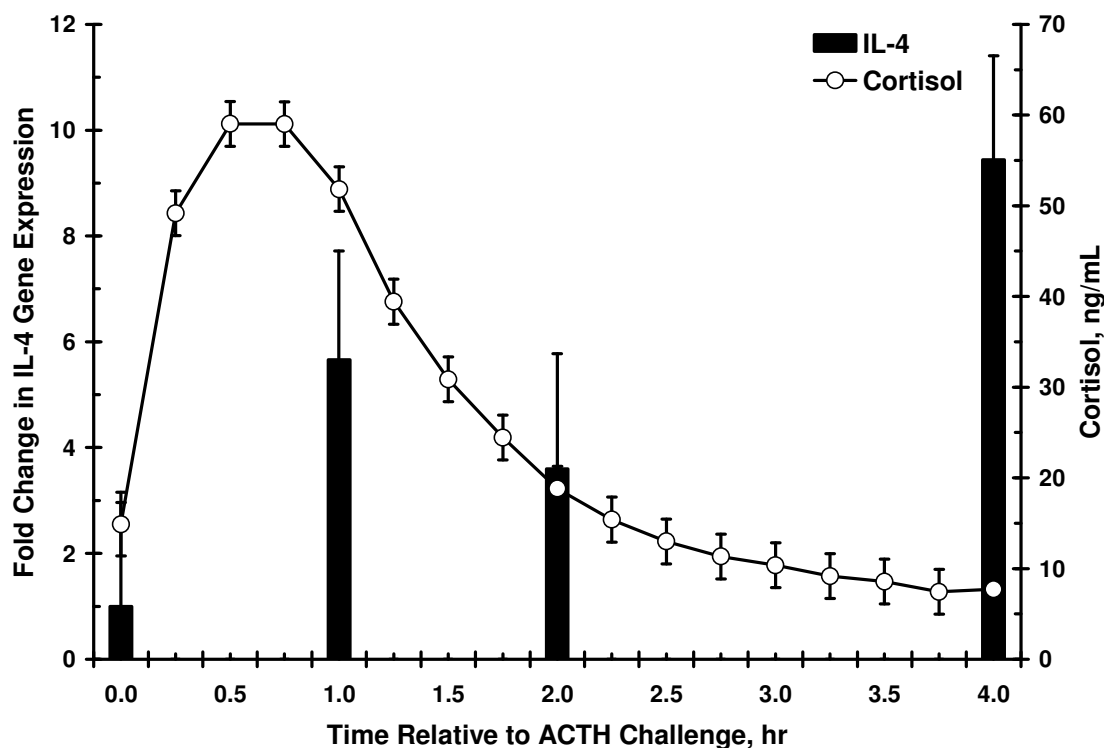


Figure 24. Fold change in gene expression of IL-4 in isolated peripheral blood mononuclear cells (PBMCs) in response to adrenocorticotropin (ACTH) challenge with data analyzed with the addition of a covariate. The covariate included in the data analysis was the time between cannulation and collection of the first sample (ranging from 0.5 to 1.25 hr). Data plotted in reference to cortisol concentrations post-ACTH challenge. Whole blood was collected and PBMCs isolated at 0, 1, 2 and 4 hr relative to a challenge with 0.1 IU/kg BW ACTH. Total RNA was extracted from isolated PBMCs and gene expression determined using quantitative real-time RT-PCR. The greatest fold increase in expression of IL-4 occurred 4 hr post-challenge ($P = 0.032$; LS Mean \pm SEM). The covariate explained some variation associated with the expression of IL-4 ($P = 0.027$).

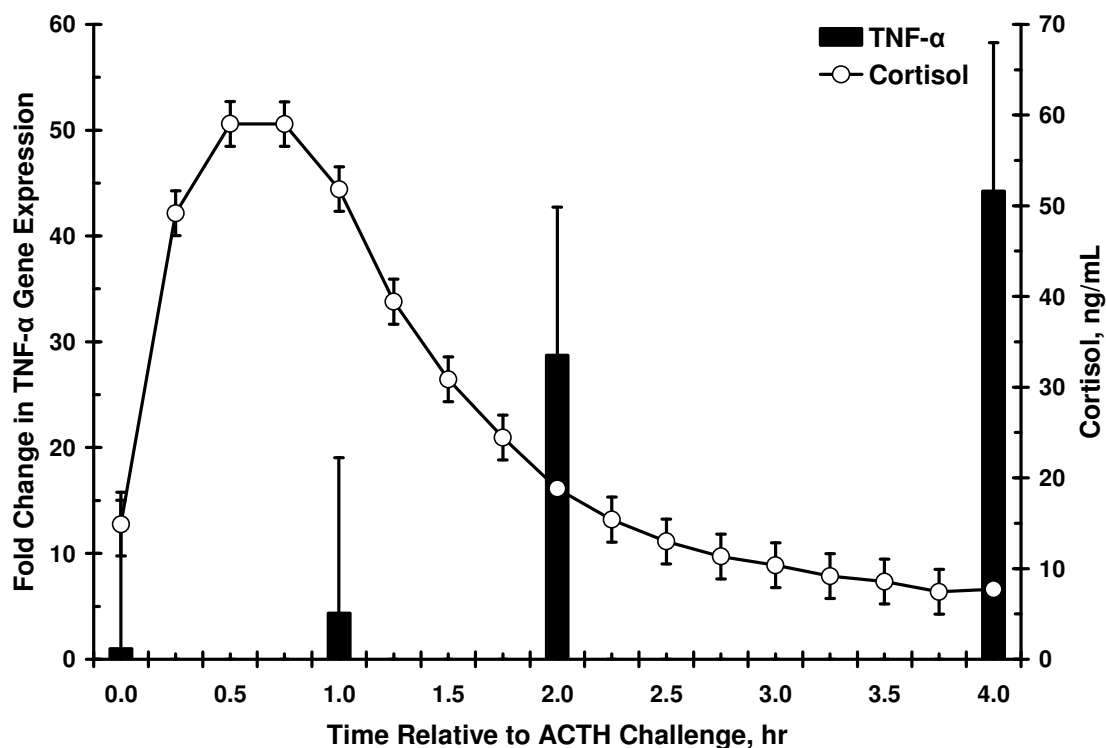


Figure 25. Fold change in gene expression of TNF- α in isolated peripheral blood mononuclear cells (PBMCs) in response to adrenocorticotropin (ACTH) challenge with data analyzed with the addition of a covariate. The covariate included in the data analysis was the time between cannulation and collection of the first sample (ranging from 0.5 to 1.25 hr). Data plotted in reference to cortisol concentrations post-ACTH challenge. Whole blood was collected and PBMCs isolated at 0, 1, 2 and 4 hr relative to a challenge with 0.1 IU/kg BW ACTH. Total RNA was extracted from isolated PBMCs and gene expression determined using quantitative real-time RT-PCR. The gene expression of TNF- α tended to increase from time 0 to 4 hr post-ACTH challenge ($P = 0.121$; LS Mean \pm SEM). Addition of the covariate tended explain variation in the expression of TNF- α $P = 0.123$).

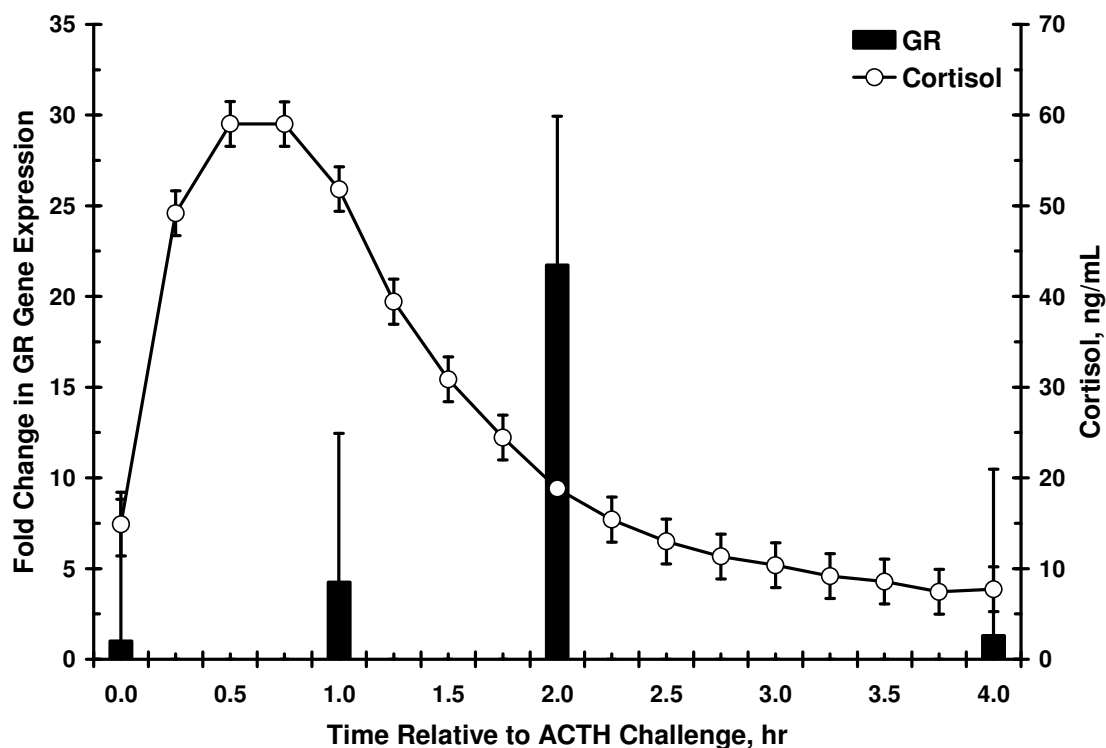


Figure 26. Fold change in gene expression of GR in isolated peripheral blood mononuclear cells (PBMCs) in response to adrenocorticotropin (ACTH) challenge, with data analyzed with the addition of a covariate. The covariate included in the data analysis was the time between cannulation and collection of the first sample (ranging from 0.5 to 1.25 hr). Data plotted in reference to cortisol concentrations post-ACTH challenge. Whole blood was collected and PBMCs isolated at 0, 1, 2 and 4 hr relative to a challenge with 0.1 IU/kg BW ACTH. Total RNA was extracted from isolated PBMCs and gene expression determined using quantitative real-time RT-PCR. The gene expression of GR did not change in response to ACTH challenge ($P = 0.270$; LS Mean \pm SEM). Addition of the covariate did not explain variation in the expression of GR ($P = 0.746$).

variability associated with IL-10 and IL-4 ($P = 0.052$ and 0.027 , respectively) but not for IFN- γ , TNF- α , or GR ($P = 0.402$, 0.0123 , and 0.746 , respectively).

DISCUSSION

These data suggest that acute increases in cortisol, such as those induced by cannulation and ACTH challenge, can increase the expression of immune (IL-10, IFN- γ , IL-4, and TNF- α) and endocrine-related (GR) genes in isolated PBMCs. Studies in rodents have demonstrated enhanced immune response to pathogens following acute stress (Dhabhar, 2000; Dhabhar, 2002, Dhabhar, 2009; Martin, 2009). However, limited studies have examined whether acute stress, in the absence of immune stimulation, can increase the expression of immune mediators. Specifically, this is the first study to demonstrate positive effects of cortisol on immunity in cattle.

Cannulation is a stressful event that requires cattle to be restrained for 5 to 10 min (Koch, 2004). Concentrations of cortisol post-cannulation were higher in the current study compared to another study utilizing Brahman cattle (26.22 ± 4.42 in current study vs. 19.71 ± 2.56 ng/mL in Koch study; Koch, 2004). The greater concentration of cortisol in the current study is probably due to the sex of the cattle as heifers are known to have greater concentrations of cortisol compared to bulls, as used by Koch (2004; Rhodes and Rubin, 1999; Burdick et al., 2009b; Welsh et al., 2009). Concentrations of stress hormones released while the animal is restrained may stimulate immune cells and the subsequent production of immune mediators (Dhabhar, 2000, Dhabhar, 2002). Additionally, cytokines and hormones released in response to the

minor tissue damage associated with the cannulation procedure may further stimulate and prepare the immune system for subsequent immune challenge. Cannulation is not a common management practice in the cattle industry; therefore, it will be necessary to determine if acute restraint in the absence of cannulation can increase expression and secretion of cytokines by PBMCs.

The administration of ACTH is used to stimulate increases in endogenous cortisol concentrations for research or pharmacologic diagnostics. In cattle, administration of 0.125 IU/kg BW ACTH resulted in an increase in cortisol concentrations within 0.5 hr, returning to baseline concentrations by 2.5 hr post-challenge (Lay et al., 1996). The ACTH dose in the current study was slightly lower, yet the temporal pattern and magnitude of response were similar to Lay et al. (1996).

As the primary mediator of inflammation, $\text{TNF-}\alpha$ is often the cytokine produced first in response to infection or tissue damage (Pfeffer, 2003). Similarly, $\text{IFN-}\gamma$ is produced by lymphocytes to activate macrophages during the inflammatory response. Alternatively, IL-4 is involved in the activation of adaptive immunity, particularly in the activation of B lymphocytes and the humoral immune response. The production of IL-10 stimulates the termination of the inflammatory response, resulting in a shift from production of pro-inflammatory to production of anti-inflammatory cytokines. Interestingly, the expression of both pro-inflammatory ($\text{TNF-}\alpha$ and $\text{IFN-}\gamma$) and anti-inflammatory (IL-4 and IL-10) cytokines was stimulated by acute increases in cortisol in response to both cannulation, but only IL-10 and IL-4 increased in response to ACTH challenge. As expression of all cytokines tended to increase in response to cannulation,

it is possible that this initial increase in gene expression prevented the production of a response to ACTH (i.e., increase in expression of IFN- γ , TNF- α , and GR). This suggests that in response to acute stress, the body prepares and arms the immune system with ammunition (in the form of cytokines) in order to potentially battle a subsequent immune challenge. However, increases in expression do not necessarily translate into increases in secreted protein and therefore it will be necessary to determine if these increases in cytokine gene expression indeed translates into an increase in secreted protein.

The GR mediates the responses to glucocorticoids at the cellular level. The mineralocorticoid receptor (MR) has greater affinity for glucocorticoids, while glucocorticoids bind to the GR only when glucocorticoids saturate the MR (Carrasco and Van de Kar, 2003; Sorrells and Sapolsky, 2007). However, the MR is not expressed in cells of the immune system. It has been suggested that the MR mediates the enhancing and priming effects of glucocorticoids, while the GR mediates the negative effects of glucocorticoids on immune function (Sorrells and Sapolsky, 2007). If that is the case, increases in expression of the GR may also serve as to prepare the body for subsequent challenge as glucocorticoids, produced in response to infection, serve to inhibit inflammation thereby preventing a hyperinflammatory state. However, Sorrells and Sapolsky (2007) also state that the dichotic effects of acute versus chronic stress may be attributed more to the extent and duration that the GR is occupied.

Glucocorticoids modulate cytokines through two mechanisms involving binding to the GR. First, through protein-protein interactions, glucocorticoids are known to inhibit the transcription factors, AP-1 and NF- κ B, which are responsible for increasing

the expression of cytokines (Ashwell et al., 2000; Guyre et al., 2008). This includes the four cytokines analyzed in the current study. Specifically, glucocorticoids enhance the production of I κ B, the inhibitor of NF- κ B (Guyre et al. 2008). Secondly, glucocorticoids decrease the stability and the half-life of cytokine-encoding mRNAs (Ashwell et al., 2000; Tsigos and Chrousos, 2002; Rhen and Cidlowski, 2005; Guyre et al., 2008). While the mechanism by which glucocorticoids and the GR inhibit the expression of cytokines is well known, the mechanism in which glucocorticoids enhance immune function is less apparent (Dhabhar, 2009). In order to truly understand the mechanisms in which acute stress enhances immune function, additional studies must be completed.

Exogenous ACTH has a short half-life and therefore should not remain in circulation for more than 40 minutes (Lay et al., 1996). However, it is possible that the effects on gene expression in the isolated PBMCs were due to direct actions of ACTH, as lymphocytes have been demonstrated to express ACTH receptors (Smith, 2008). Currently it is unclear if ACTH has direct effects on isolated PBMCs and if ACTH and cortisol work in synergistic or additive manner to increase expression of immune and endocrine-related genes in peripheral blood leukocytes.

Addition of the covariate to the analysis (time between cannulation and collection of the first sample) accounted for variation in the production cortisol (both pre- and post-ACTH challenge), and in the gene expression of IL-4 and IL-10 in response to ACTH. This suggests that the timing between acute stressors, in this case the timing between cannulation and subsequent ACTH challenge, may influence the production of cortisol and the expression of cytokines. As stated earlier, no significant

changes in the gene expression of IFN- γ , TNF- α , or the GR occurred in response to ACTH; however, all three genes increased, or tended to increase in response to cannulation. Therefore, the gene expression of our genes of interest in response to ACTH challenge may have been confounded by the already elevated gene expression levels induced by cannulation. In order to elucidate the pattern of change in gene expression in response to these acute challenges, the challenges need to be separated with enough time as to prevent one challenge from affecting the other. Additionally, this data suggests that the timing between cannulation and the onset of a challenge in which stress or immune mediators are measured needs to be taken into consideration during experiment planning.

Cortisol administered 144 hr or less prior to an immune challenge has been demonstrated to enhance the immune response (Besedovsky et al., 1996; Sapolsky et al., 2000; Sorrells and Sapolsky, 2007). Therefore, the increase in expression of cytokines in response to cannulation- and ACTH-induced increases in endogenous cortisol may be beneficial if the animals are subsequently exposed to a pathogen. In conclusion, these data suggest a positive effect of cortisol on immune function in cattle. While studies in rodents and humans have demonstrated enhanced immunity following exposure to acute stress, this has yet to be demonstrated in larger livestock species. Additional studies are required to determine if these changes enhance the immunologic response to subsequent immune challenge following acute stress (e.g., handling, transportation, weaning) in cattle.

CHAPTER VII

GENERAL DISCUSSION

This project was comprised of a series of studies designed to investigate the influence of specific stressors on Brahman cattle, and whether temperament affected the responsiveness of the cattle to the stressors. Temperament was determined using objective (exit velocity) and a subjective (pen score) scoring methods, to assign each animal a temperament score. Based on temperament score calves were ranked as Calm (lowest scores), Temperamental (highest scores), or Intermediate (middle scores). Specifically, results of these studies demonstrate that temperament differentially affects the physiologic, endocrinologic, and immunologic responses to exposure to an endotoxin, to transportation events, or to exogenous ACTH. Additionally, the duration of the stressor may influence whether it negatively or positively affects aspects of the immune system.

Two transportation studies were conducted. In the first transport study (Chapter III), in which cattle were transported by truck and trailer for 9 hr, transportation influenced the rectal temperature response, but not the stress hormone responses (cortisol and epinephrine), to transportation. We recognize that potentially, during the first transport study, stress factors may have changed during transportation when samples were not collected. This has previously been suggested by Blecha et al. (1984) who similarly did not find changes in cortisol concentrations before and after a 10 hr transport of Angus and Angus-Brahman cross steers. Another factor that may have

prevented finding changes in peripheral blood concentrations of stress hormones due to transportation may be the manner in which samples were collected (i.e., blood sampling protocol). Blood samples were collected at cannulation prior to loading the cattle onto the trailer (pre-transport samples) and after transportation following unloading of the cattle out of the trailer (post-transportation samples). Therefore, the blood concentrations of cortisol and epinephrine may have been influenced more by the cattle handling procedure than the actual transport, thus finding no differences in the stress hormone concentrations in the pre- and post-transport samples.

The second transportation study (Chapter V), was designed in order to alleviate issues encountered in the earlier study (Chapter III). Automatic sampling devices were programmed to collect blood samples prior to and during transportation in the absence of humans. Additionally, cattle remained on the trailer for 2 hr prior to transportation, during which samples were collected, in order to separate the response to loading into the trailer and the response to transportation. There were clear differences in the parameters measured in both studies due to the changes in the sampling protocol for the later study. First, rectal temperature was not affected by transportation, which is different from the earlier study which found an increase in rectal temperature within the first 0.5 hr of transportation. Ambient temperatures also increased during the later study, and were highly correlated with rectal temperature in both Calm and Temperamental bulls. Although rectal temperature in the later study increased throughout the study, this increase was not due to transportation and was not affected by temperament. This suggests that the temperature response observed during the first transportation study may

have been a result of loading and immediate transportation, as the calves in the second study that were rested prior to transportation did not exhibit a similar response. This concurs with Odore's et al. (2004) suggestion that loading and unloading seem to be more stressful than the actual transportation event when blood cortisol was used as an indicator of stress responsiveness. Furthermore, in our study, transportation only influenced cortisol concentrations, a response that was solely exhibited by Calm calves.

In the studies reported in this dissertation, temperament has been demonstrated to have a greater influence on parameters measured than the actual stressor applied. With respect to the transportation studies, temperament influenced rectal temperature, cortisol and epinephrine responses prior to and after transportation (Chapter III). Additionally, when blood samples were collected during transportation, only Calm calves demonstrated a cortisol response to transportation. In response to LPS challenge, the epinephrine response was affected by temperament, in which Calm and Temperamental bulls elicited a response, while Intermediate bulls did not. However, the cortisol response to LPS was not affected by temperament; although pre-LPS cortisol concentrations were greater in Temperamental bulls compared to Calm bulls. Additionally, Temperamental bulls had greater basal rectal temperature prior to transportation and LPS challenge. Sickness behavior of calves in response to LPS was also affected by temperament, in that Calm calves exhibited more sickness behaviors than Temperamental cattle.

The type of stressor differentially affects both physiological and endocrine parameters. While Temperamental bulls had greater basal rectal temperature prior to

transportation (Chapter III) and LPS challenge (Chapter IV), their temperature responses to the subsequent challenges were different. Specifically, rectal temperature in all bulls increased in response to transportation before decreasing, with Temperamental bulls maintaining a rectal temperature similar to Intermediates, but greater than Calm bulls, throughout the remainder of the study. In contrast, the increase in rectal temperature in response to LPS challenge was smaller in Temperamental bulls when compared to the increase in rectal temperature by Calm and Intermediate bulls.

The differences in rectal temperature responses to transportation and LPS challenge can have important implications for the livestock industry. Cattle may be subjected to transportation multiple times during production, and are often comingled with different cattle upon their arrival. Cattle from different sources may carry pathogens that are foreign to other cattle, therefore increasing their risk of disease. Often, it is an elevated rectal temperature that is used to diagnose and ultimately determine if an animal should be treated. Therefore, understanding the changes in rectal temperature in response to transportation versus those in response to a pathogen may be important in determining which cattle should be treated upon arrival.

There was limited influence of transportation on immune function immediately following the end of transportation (Chapter V). Previous studies have demonstrated negative effects of transportation on immune function in cattle (Blecha et al., 1984; Murata and Hirose, 1991; Buckham Sporer et al., 2007, 2008). However, in those studies samples were collected several hours or days following the end of transport. In the present study samples were only collected immediately after the cessation of

transportation. Therefore, future studies will need to study the effects of transportation on immune function utilizing samples collected several hours to days following the cessation of transportation.

While glucocorticoids seem essential to prevent hyperactivity of the immune system, under basal conditions glucocorticoids act in a permissive and preparatory manner, allowing for quick action against an invading pathogen (Guyre et al., 2008). In the final study reported in this dissertation (Chapter VI), cortisol concentrations induced by cannulation and ACTH increased the expression of cytokines in isolated PBMCs. Therefore, if the acute increase in cortisol due to these processes induced the increase in cytokine gene expression, it demonstrates that acute increases in cortisol can be beneficial to the health of cattle.

CHAPTER VIII

CONCLUSIONS AND IMPLICATIONS

Different stressors are known to modulate physiological, endocrine, and immune parameters. The temperament of cattle may also influence the response to various stressors. These studies demonstrate that transportation is not equally stressful across temperament groups. Additionally, loading and unloading cattle from a trailer may have a greater influence on physiological and endocrine parameters than the actual transportation event. Furthermore, while temperamental cattle have greater basal concentrations of stress hormones, LPS challenge produced a differential response on the adrenal gland by influencing epinephrine but not cortisol concentrations. Also, Calm and Intermediate calves displayed more behavioral signs of sickness compared to Temperamental cattle. Lastly, acute increases in cortisol, or cortisol concentrations elevated for a short period of time, may have beneficial actions on the immune system by potentially preparing the body for a subsequent immune challenge.

Ultimately, these studies suggest that 1) a rest period prior to transportation may alleviate the stress associated with transportation; 2) cattle with more excitable temperaments may not display similar physiological or endocrine signs of sickness compared to calm cattle, and perhaps are protected from the detrimental effects of LPS due to greater basal concentrations of stress hormones, and 3) acute increases in cortisol may have positive effects on immune function. The information on the response of

cattle to these stressors can ultimately be used to modify cattle management practices in order to:

- 1) alleviate stress,
- 2) minimize negative influences of illness on production (growth, and carcass characteristics), and
- 3) target application of acute stressors to enhance immune function and overall cattle health.

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APPENDIX A
CORTISOL RADIOIMMUNOASSAY PROTOCOL

Cat. No. TCO1 928, Diagnostic Products Corporation (Siemens, Los Angeles, CA)

1. Label appropriate number of tubes for assay. Include tubes for total counts, NSB, standards, controls in singles, and samples in duplicate and store covered at 4°C overnight in the refrigerator.
2. Thaw samples overnight at 4°C in the refrigerator.
3. On day of assay, set out tracer (in the hood), standards and tubes (on benchtop) to allow to warm to room temperature.
4. Dilute the 1 µg/dL calibrator (standard A) in 2 separate microcentrifuge tubes to a concentration of 0.5 and 0.25 µg/dL using the calibrator
5. Pipet 25 µL of the calibrator A into NSB and Standard A tubes.
6. Pipet 25 µL of remaining calibrators (including 2 newly diluted standards in step 4) into appropriate tubes. Be sure to pipet sample directly into the bottom of the tube.
7. Lay absorbent diapers over the edge of the hood. Pour cortisol I¹²⁵ tracer into the clear cup labeled I¹²⁵. If cup is dirty, rinse with RO water and pour rinse in waste bin.
8. Carefully pipet 1 mL of tracer into every tube. Avoid splashing solution out of the tube. This step must be completed within 10 minutes. Remove total count tube(s) and set aside.

9. Cover tubes with parafilm and incubate tubes for 45 min in a 37°C water bath.
10. Discard parafilm in radioactive waste container and move tubes into foam racks.

Decant the tubes into the waste container in the radioactive hood. Tap tubes on absorbent towel to remove residual liquid and allow to sit upside down for at least 10 minutes.

11. Tap tubes again on absorbent towel to remove residual liquid and move tubes to the gamma counter racks for counting.
12. Count the tubes using a gamma counter and the appropriate program for measuring I^{125} .

APPENDIX B

BI-CATECHOLAMINE ENZYME IMMUNOASSAY PROTOCOL

Cat. No. 17-BCTHU-E02, Alpcos Diagnostics, Boston, MA

A. Extraction

1. Allow all reagents to warm to room temperature, and samples to thaw on ice.
2. Dilute the 50 mL Wash Buffer Concentrate with DI water to a final concentration of 500 mL and store at 4°C.
3. Pipette 10 µL of standards and controls in duplicate in appropriate wells on the microtiter plate. Add 250 µL of DI water to these wells to correct for volume. Pipette 300 µL of plasma into appropriate wells in singlets.
4. Pipette 50 µL of Assay Buffer into all wells.
5. Pipette 50 µL of Extraction Buffer into all wells.
6. Cover the plate with adhesive foil and incubate 30 min at room temperature on plate shaker (600 rpm).
7. Remove the foil and discard. Immediately decant the plate and remove residual liquid by tapping the inverted plate on a paper towel.
8. Pipette 1 mL of diluted Wash Buffer Concentrate into all wells. Cover with adhesive foil and incubate for 5 min at room temperature on plate shaker.
9. Repeat step 7 and 8.
10. Repeat step 7. Pipette 150 µL of Acylation Buffer into all wells.
11. Pipette 25 µL of Acylation Reagent into all wells.

12. Incubate the plate without foil for 15 min at room temperature on plate shaker.
13. Decant the plate immediately and remove residual liquid.
14. Pipette 1 mL of diluted Wash Buffer Concentrate into all wells.
15. Incubate the plate with foil for 10 min at room temperature on plate shaker.
16. Decant the plate immediately and remove residual liquid.
17. Pipette 150 μ L of Hydrochloric Acid into all wells to elute epinephrine and norepinephrine.
18. Cover the plate with adhesive foil and incubate for 10 min at room temperature on the plate shaker.

B. Epinephrine Assay

1. Prepare Enzyme Solution no more than 10-15 minutes in advance.
Reconstitute the vial labeled Enzyme with 1 mL DI water and mix thoroughly. Add 0.3 mL of Coenzyme followed by 0.7 mL of Enzyme Buffer.
2. Remove Epinephrine 96-well ELISA plate and pipette 25 μ L of freshly-prepared Enzyme Solution into all wells.
3. Pipette 100 μ L of the extracted standards, controls, and samples into appropriate wells.
4. Incubate for 30 min at room temperature on plate shaker (400 rpm).
5. Pipette 50 μ L of Epinephrine Antiserum into all wells.

6. Cover with adhesive foil and incubate for 2 hr at room temperature on plate shaker.
7. Wash plate 3 times with diluted Wash Buffer Concentrate using a plate washer. Blot dry by inverting plate on paper towel.
8. Pipette 100 μ L of the Enzyme Conjugate into all wells.
9. Incubate for 30 min at room temperature on plate shaker.
10. Wash plate 3 times with diluted Wash Buffer Concentrate using a plate washer. Blot dry by inverting plate on paper towel.
11. Pipette 100 μ L of Substrate into all wells.
12. Cover with foil to avoid exposure to light and incubate for 30 min at room temperature on plate shaker.
13. Add 100 μ L of Stop Solution to each well and shake the microtiter plate to ensure a homogeneous distribution of the solution.
14. Read the absorbance within 10 min using a spectrophotometer set to 450 nm and a reference wavelength of 630 nm.

C. Norepinephrine Assay

1. Prepare Enzyme Solution no more than 10-15 minutes in advance.
Reconstitute the vial labeled Enzyme with 1 mL DI water and mix thoroughly. Add 0.3 mL of Coenzyme followed by 0.7 mL of Enzyme Buffer.
2. Remove Epinephrine 96-well ELISA plate and pipette 25 μ L of freshly-prepared Enzyme Solution into all wells.

3. Pipette 20 μL of the extracted standards, controls, and samples into appropriate wells.
4. Incubate for 30 min at room temperature on plate shaker (400 rpm).
5. Pipette 50 μL of Norepinephrine Antiserum into all wells.
6. Cover with adhesive foil and incubate for 2 hr at room temperature on plate shaker.
7. Wash plate 3 times with diluted Wash Buffer Concentrate using a plate washer. Blot dry by inverting plate on paper towel.
8. Pipette 100 μL of the Enzyme Conjugate into all wells.
9. Incubate for 30 min at room temperature on plate shaker.
10. Wash plate 3 times with diluted Wash Buffer Concentrate using a plate washer. Blot dry by inverting plate on paper towel.
11. Pipette 100 μL of Substrate into all wells.
12. Cover with foil to avoid exposure to light and incubate for 30 min at room temperature on plate shaker.
13. Add 100 μL of Stop Solution to each well and shake the microtiter plate to ensure a homogeneous distribution of the solution.
14. Read the absorbance within 10 min using a spectrophotometer set to 450 nm and a reference wavelength of 630 nm.

APPENDIX C
CREATININE ENZYME IMMUNOASSAY PROTOCOL

1. Allow all reagents to come to room temperature.
2. Mix the standard by adding 15 μL of the 50 mg/dL standard with 360 μL DI water.
3. Pipet 30 μL of standard, pool and samples in duplicate to appropriate wells in a 96 well plate.
4. Mix Working Solution by adding 10.25 mL of Working Reagent A and 10.25 mL of Working Reagent B to a 50 mL conical tube. Mix new working solution for each assay.
5. Add 200 μL of Working Solution to each well quickly with a multichannel pipette.
6. Incubate for 1 min and read absorbance with a plate reader at 490 nm.
7. Incubated 4 min and read absorbance at 490 nm.
8. To calculate concentration of Creatinine in each sample, first take the mean absorbance from steps 6 and 7 for the standard, pool, and samples. Second, subtract the mean absorbance value from step 6 from the mean absorbance value from step 7. Lastly, divide the difference in absorbance values calculated for each sample by the difference in absorbance values calculated for the standard.

APPENDIX D

ISOLATION OF BOVINE PERIPHERAL BLOOD MONONUCLEAR CELLS FROM
WHOLE BLOOD, CULTURE AND PROLIFERATION ASSAY PROTOCOL

Proliferation Kit: Cat No. G4101; Promega (Madison, WI)

1. Mix PBMC media by adding 44 mL DME/F12 (Cat No. 1330-032; Invitrogen, Carlsbad, CA), 5 mL Horse Serum (heat inactivated; Cat No. 16777-030; VWR, West Chester, PA), 0.5 mL penicillin/streptomycin (Cat No. 15140-122; Invitrogen, Carlsbad, CA), 0.5 mL L-glutamine (Cat No. 25030-081; Invitrogen, Carlsbad, CA), and 3.52 μ L 2-mercaptoethanol (diluted 1:100 in DME/F12; Cat No. M7522; Sigma Aldrich, St. Louis, MO) per 50 mL of media needed. Sterile filter through a 0.2 μ m filter and store at 4°C in a refrigerator.
2. Dilute ConA (Cat No. L7647; Sigma Aldrich; St. Louis, MO) from a stock concentration of 5 mg/mL to 20 μ g/mL using prepared media. Serial dilute the 20 μ g/mL ConA dose to 10, 5, 2.5, 1.25, 0.625, 0.3125 μ g/mL using prepared media. Add 50 μ L of prepared ConA doses (0-20 μ g/mL) in triplicate to appropriate wells in a 96-well culture plate (flat bottom). Place plates in incubator.
3. Collect one 7-mL tube of whole blood in a vacutainer containing EDTA.
4. Dilute the whole blood in 10 mL of 1x Hank's Balanced Salt Solution (HBSS; Cat No. 14185-052; Invitrogen, Carlsbad, CA) in a 50-mL conical tube.
5. Add 5 mL of Ficoll-Paque (Cat No. 17-1440-03; VWR, West Chester, PA) to 2 15-mL conical tubes. Layer 8.5 mL of diluted whole blood on top of Ficoll.

6. Centrifuge at 2300 rpm for 30 min at room temperature with no brake.
7. Collect buffy coat layer from both 15-mL tubes and place in a new 50-mL conical tube.
8. Add HBSS to the 40-mL mark on the conical tube.
9. Centrifuge at 1300 rpm for 10 min at 4°C.
10. Aspirate supernatant and lyse any red blood cells with 5 mL 0.2% NaCl and mix by trituration. Quickly add 5 mL 1.6% NaCl and add HBSS to the 40-mL mark on the conical tube.
11. Centrifuge at 1300 rpm for 10 min at 4°C.
12. Aspirate supernatant and resuspend isolated PBMCs in prepared media.
13. Determine concentration of PBMCs using a hemocytometer, diluting PBMCs 1:50 using trypan blue (Cat No. 82024-256; VWR, West Chester, PA).
14. Resuspend PBMCs to a final concentration of 1×10^7 cells in 5 mL of prepared media.
15. Add 50 μ L of final resuspension of PBMCs to prepared culture plate in triplicate.
16. Incubate cells for 96 hr at 37°C and 5% CO₂.
17. To determine proliferation of cells, add 15 μ L of MTT dye to each well following incubation for 92 hr. Place plates back in incubator for 4 hr.
18. Add 100 μ L of Stop Solution to each well. Place in a tuperware box with a moist paper towel overnight. Read the plate absorbance at 562 nm.
19. In duplicate plates for IgM production, seal plates with a plate sealer and store in a freezer at -20°C.

APPENDIX E

BOVINE IgM ELISA PROTOCOL TO MEASURE IgM SECRETED BY CULTURED
PBMCs

Bovine IgM ELISA kit: Cat No. E10-101; Bethyl Laboratories, Montgomery, TX

A. Buffers

1. Coating Buffer

- i. Add 2.12 g Na_2CO_3 to 150 mL of DI water and stir until dissolved
- ii. Adjust pH to 9.6 using HCl.
- iii. Add DI water to a final volume of 200 mL. Store at room temperature.

2. 10x TRIS Solution

- i. In 800 mL DI water add 60.7g TRIS-base and 81.8g NaCl and stir until dissolved.
- ii. Adjust pH to 8.0 using HCl and add DI water to a final volume of 1000 mL. Store at room temperature

3. Wash Solution

- i. Mix 100 mL of 10x TRIS Solution with 900 mL DI water.
- ii. Add 0.5 mL Tween20 slowly, as it is very viscous. Stir until mixed.
Store at room temperature

4. Blocking Buffer

- i. Mix 10 mL of 10x TRIS Solution and 80 mL DI water.

- ii. Add 1 g bovine serum albumin and on top of solution and let sit to dissolve. Do not mix.
- iii. Add DI water to a final volume of 100 mL.
- iv. Optional: sterile filter using a 0.45 nm filter and store at 4°C

5. Sample Diluent

- i. Add 1 g bovine serum albumin on top of 90 mL Wash Solution and let sit to dissolve. Do not mix.
- ii. Add DI water to a final volume of 100 mL.
- iii. Optional: sterile filter using a 0.45 nm filter and store at 4°C.

6. Enzyme Substrate (ABTS)

- i. Mix a 0.2M dibasic NaPO_4 solution by adding 14.196 g dibasic NaPO_4 to 500 mL of DI water.
- ii. Mix a 0.1M citric acid solution by adding 10.505g citric acid to 500 mL of DI water.
- iii. Add 1 ABTS tablet to 25.7 mL 0.2M dibasic NaPO_4 and 24.2 mL 0.1M citric acid and let dissolve.
- iv. Adjust pH to 4.5 mL using HCl.
- v. Add DI water to a final volume of 100 mL.
- vi. Store in 14 mL aliquots labeled ABTS at -20°C.

B. Bovine IgM Standards

- 1. Add 4 μL of Bovine IgM Reference Serum to 10 mL of sample diluent to make a standard concentration of 1000 ng/mL. Serial dilute to create 500,

250, 125, 62.5, 31.25, and 15.625 ng/mL standards. Aliquot into 1-mL aliquots in microcentrifuge tubes and store at -20°C.

C. Bovine IgM ELISA

1. Coat plate with primary (coating) antibody
 - i. Dilute primary antibody 1:100 in Coating Buffer
 - ii. Add 100 μ L per well of a 96-well plate
 - iii. Cover plate with parafilm and incubate for 1 hr on plate shaker at room temperature.
 - iv. Incubate overnight at 4°C.
 - v. Warm up plate for 30 min on plate shaker at room temperature.
 - vi. Wash plate 3 times with Wash Buffer using plate washer.
2. Add Blocking Buffer
 - i. Add 200 μ L Blocking Buffer to each well.
 - ii. Cover plate with parafilm and incubate for 1 hr on plate shaker at room temperature.
 - iii. Wash plate 3 times with Wash Buffer using plate washer.
3. Add Standards and Samples
 - i. Add 100 μ L of standards in triplicate to appropriate wells.
 - ii. In sample wells, add 90 μ L of sample diluent.
 - iii. Add 10 μ L of sample to sample wells (in triplicate).
 - iv. Cover with parafilm and incubate for 2 hr on plate shaker at room temperature.

- v. Wash plate 4 times with Wash Buffer using a plate washer.
- 4. Add secondary (detecting) antibody
 - i. Dilute secondary antibody 1:3200 in sample diluent.
 - ii. Add 100 μ L to each well.
 - iii. Cover with parafilm and incubate for 1 hr on plate shaker at room temperature.
 - iv. Place ABTS in 37°C water bath to thaw.
 - v. Wash plate 4 times with Wash Buffer using a plate washer.
- 5. Add enzyme substrate (ABTS)
 - i. Add 5 μ L 30% H_2O_2 per tube of ABTS and mix gently.
 - ii. Add 100 μ L ABTS per well and cover with parafilm.
 - iii. Incubate plate in the dark for 30 min at room temperature.
 - iv. Read the absorbance at 405 nm using a spectrophotometer.

APPENDIX F

PERIPHERAL BLOOD MONONUCLEAR CELL RNA EXTRACTION PROTOCOL

USING INVITROGEN PURELINK MICRO TO MIDI TOTAL RNA

PURIFICATION SYSTEM PROTOCOL

Cat No. 12183-018; Invitrogen, Carlsbad, CA

1. Add 60 mL of 100% ethanol to Wash Buffer II from new kit.
2. Thaw PBMC samples on wet ice. Centrifuge for 10 minutes at room temperature at 1.4 x G using a microcentrifuge. Remove supernatant and discard.
3. Mix fresh Lysis Solution by adding 10 μ L of 2-mercaptoethanol for every 1 mL Lysis Solution needed. Determine the amount of Lysis solution needed by multiplying 600 μ L by the number of samples plus 1. For example, for 15 samples multiply 600 by 16.
4. Add 600 μ L of freshly made Lysis Solution to each sample and vortex well.
5. Homogenize samples by passing samples through a 20-gauge needle attached to a 1-cc nuclease-free syringe 10 times.
6. Add 600 μ L of 70% ethanol to each sample. Mix by vortexing and disperse any precipitate that may have formed. Be sure to complete steps 4-6 on one sample before moving onto next sample.
7. Transfer 700 μ L of sample to a RNA spin cartridge pre-inserted into a collection tube. Centrifuge at 13,000 x G for 20 sec at room temperature. Discard flow through and re-insert cartridge into tube.

8. Repeat step 7 until all sample has been added.
9. Add 700 μL of Wash Buffer I to spin cartridge. Centrifuge at 13,000 x G for 20 sec at room temperature. Discard flow through and collection tube.
10. Place spin cartridge in new Wash Tube.
11. Add 500 μL of Wash Buffer II (with ethanol) to spin cartridge. Centrifuge at 13,000 x G for 20 sec at room temperature. Discard flow through and re-insert cartridge into the tube.
12. Repeat step 11 once.
13. Centrifuge spin cartridge at 13,000 x G for 1 min at room temperature to dry membrane with attached RNA. Discard the collection tube and insert the cartridge into an RNA recovery tube.
14. To elute RNA, add 30 μL RNase-free water to the center of the spin cartridge and incubated at room temperature for 1 min.
15. Centrifuge the spin cartridge for 2 min at 13,000 x G at room temperature.
16. Discard spin cartridge and place the samples on ice.
17. Determine concentration of samples using a NanoDrop spectrophotometer.
 - a. Bring blank (2 μL of nuclease-free water), your P2 pipette and tips, and your samples (undiluted).
 - b. Click on “Default.”
 - c. Click on “Nucleic Acid.”
 - d. Clean the 2 holes (top and bottom) with provided squirt bottle, “Nano H₂O,” and wipe with provided kimwipe.

- e. Load 2 μL of provided sterile water onto bottom hole, close the top lever and click “Calibrate.”
- f. Listen for clicking noise and wait for screen to clear.
- g. In between samples wipe top and bottom holes with kimwipe.
- h. For blank, pipette 1 μL on bottom hole, close the top lever, and click “Blank.” Look for 0.00 wavelength.
- i. Wipe top and bottom holes with kimwipe.
- j. Click “sample type” (RNA-40).
- k. To print click on “start report” button and type your sample i.d.
- l. Pipette 1 μL of your stock sample onto bottom hole, lower the top lever, and click “measure.” Look at the graph on the screen. If your sample curve extends beyond then you need to dilute your sample 1:10.
- m. Wipe top and bottom holes with kimwipe.
- n. To print you must type in an i.d. for each sample that you click “measure.”
- o. To clean up: wipe top and bottom holes with provided “Nano H_2O .”
- p. Replace pad in between the 2 holes.
- q. To view report click on “show report” button.
- r. To print click “print.”

18. Samples should be stored in a -80°C freezer.

APPENDIX G

DNASE TREATMENT AND cDNA SYNTHESIS PROTOCOL

DNase I Amplification Grade kit: Cat No. 18083-015; Invitrogen, Carlsbad, CA

Superscript III First-Strand Synthesis System for RT-PCR kit: Cat No. 18080-051;

Invitrogen, Carlsbad, CA

1. Calculate the volume of isolated RNA required to make a 1 μg concentration in a total volume of 20 μL by dividing 1 by the total concentration of RNA in each sample and multiplying by 1000.
 - a. Example: if RNA concentration is 100 ng/ μL , divide 1 by 2.5 and multiply by 1000, which will give the μL of sample $(1 \div 100) \times 1000 = 10 \mu\text{L}$.
2. Determine the amount of nuclease-free water needed to dilute the sample to a 1 μg concentration by subtracting the volume of isolated RNA calculated in step 1 from 20 μL .
 - a. Example: Subtract 10 μL of sample (previous example) from 20 μL will give you 10 μL of nuclease-free water needed to dilute sample.
3. Dilute the RNA according to the volumes of isolated RNA and nuclease-free water determined in steps 1 and 2 in a nuclease-free microcentrifuge tube and place on ice.
4. Mix DNase Treatment Master Mix by adding 2.4 μL of 10x DNase Buffer with 1 μL of DNase I per reaction needed to a nuclease-free microcentrifuge tube and mix well by pipette. When determining the number of reactions, include one reaction for each

sample plus one extra reaction. For example, when there are 2 samples, make a master mix for 3 reactions. Place master mix on ice.

5. Add 3.4 μL of the DNase Treatment Master Mix to each diluted sample and mix well by triturating or flicking the tube by hand. Do not vortex!
6. Incubate samples at room temperature for 15 min.
7. Add 2.4 μL of 25 mM EDTA to each sample and mix well by triturating or flicking the tube.
8. Incubate samples at 65°C for 10 min using a heat block. Place samples on ice.
9. Mix 1st Strand Synthesis Master Mix by adding 2 μL of OligodT and 2 μL of dNTPs per reaction needed to a nuclease-free microcentrifuge tube and mix well. Place master mix on ice
10. Add 4 μL of the 1st Strand Synthesis Master Mix to each sample and mix by triturating or flicking the tube.
11. Incubate samples at 65°C for 5 min using a heat block. Place samples on ice for 1 min.
12. Mix cDNA Master Mix by adding 4 μL 10x RT buffer, 8 μL 25 mM MgCl_2 , 4 μL 0.1 M DTT, 2 μL RNase Out, and 2 μL Superscript III per reaction needed to a nuclease-free microcentrifuge tube and mix well. Place master mix on ice.
13. Add 20 μL of cDNA Master Mix to each sample and mix by flicking the tube.
14. Incubate at 50°C for 50 min using a water bath.
15. Incubate at 85°C for 5 min using a heat block.
16. Place samples on ice or store in a -80°C freezer.

APPENDIX H

REAL TIME QUANTITATIVE RT-PCR PROTOCOL

1. Resuspend lyophilized primers in nuclease-free water to a concentration of 100 μM .
To determine the volume of nuclease-free water to add to reach this concentration, multiply the nMol of the primer (indicated on the primer tube) by 10. The resulting number is the amount of nuclease-free water to add to the lyophilized primer. Mix well.
2. Dilute the resuspended primers 1 to 10 with nuclease-free water in a new nuclease-free microcentrifuge tube to make a working concentration of 10 μM . For example, dilute 10 μL of 100 μM primer with 90 μL of nuclease free water.
3. Dilute cDNA 1:50 with nuclease-free water in a nuclease-free microcentrifuge tube and place on ice. For example, dilute 2 μL cDNA with 98 μL nuclease-free water.
4. Mix the PCR Master Mix in a new nuclease-free microcentrifuge tube by adding 12.5 μL 2x SYBR Green Master Mix (Cat No. 4309155; Applied Biosystems, Foster City, CA), 0.625 μL of 10 μM forward primer, 0.625 μL of 10 μM reverse primer, and 1.25 μL of nuclease-free water to a nuclease-free microcentrifuge tube, for a total volume of 15 μL per sample, and keep on ice. When determining the number of reactions, include one reaction for each sample plus one extra reaction. For example, when there are 2 samples, make a PCR Master Mix for 3 reactions.
5. Add 15 μL of the mixed PCR Master Mix to the appropriate wells of a 96 clear well PCR Plate (Cat No. 4346906; Applied Biosystems, Foster City, CA) that is sitting on

top of ice. Add 10 μ L of each sample in triplicate to the appropriate wells. Cover the PCR plate with a clear plate cover (Cat No. 4311971; Applied Biosystems, Foster City, CA).

6. Create a new template for absolute quantification in the SDS 1.3 program for the 7900HT Fast Real-Time PCR System. The cycle settings should be:
 - a. 1st stage: 50°C for 15 sec.
 - b. 2nd stage: 95°C for 10 min.
 - c. 3rd stage: 40 cycles of 95°C for 15 sec and 56°C for 1 min.
 - d. 4th stage (disassociation state): 95°C 15 sec and 60°C for 15 sec.

VITA

Nicole Cassandra Burdick received her Bachelor of Science degree in agriculture in animal science from Texas A&M University-Kingsville in 2005. She remained at Texas A&M University-Kingsville and received her Master of Science degree in animal science in 2007 under the direction of Dr. Jamie Laurenz. Following completion of her degree Nicole joined the Physiology of Reproduction section with the Department of Animal Science at Texas A&M University in College Station in 2007 and received her Doctor of Philosophy degree in August 2010. Nicole's interests include interactions between stress, cattle temperament, and immune function, as well as the effects of fetal programming on endocrine and immune systems. She plans to continue studying in these areas as she joins the USDA-ARS Livestock Issues Research Unit in a post-doctoral research associate position.

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